

**The Role and Regulation of PAK Isoforms in
Cancer Cell Migration**

by

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Abstract

p21-activated kinases (PAKs) are downstream targets of many Rho GTPases. They have been implicated as regulators of cell migration but little is known of their isoform-specific functions. PAK upregulation in some cancers suggests that they could play an important role in invasion and metastasis, making them potential therapeutic targets. In this study RNA interference (RNAi) was used to investigate the roles of PAK1, PAK2 and PAK4 in DU145 and PC3 prostate cancer cells in response to hepatocyte growth factor (HGF), which stimulates cell migration.

RNAi knockdown of PAK1, PAK2, or PAK4 did not affect the speed of DU145 cell wound healing *in vitro*, but knockdown of PAK1 or PAK2 had distinct effects on DU145 morphology and scattering. PAK1-deficient cells remained more spread and maintained more junctions than control cells following HGF stimulation. PAK2-deficient cells formed disorganised colonies and larger lamellipodia were observed after HGF treatment, which were enriched with ERK1/2 and GIT1/2. Despite the cytoskeletal effects, PAK1 or PAK2 knockdown did not inhibit DU145 cell migration through transwells or invasion through Matrigel, and phosphorylation of many known PAK1/2 substrates was unaffected. In PC3 cells, knockdown of PAK1 or PAK2 but not PAK4 slowed the speed of migration and PAK2 impaired the HGF-induced phosphorylation of Op18 suggesting that decreased microtubule stability may lead to the associated phenotype. PAK2 depletion in DU145 or PC3 cells led to increased phosphorylation of PAK1 at S199/204, an autophosphorylation site which blocks PIX binding, whereas PAK1 knockdown had no effect on the phosphorylation of PAK2 at the homologous sites.

These results show that PAK1, PAK2 and PAK4 play distinct roles in cytoskeletal regulation, probably through shared and isoform-specific substrates. Op18 is shown to be a likely PAK2 target and a novel regulatory mechanism from PAK2 to PAK1 is identified for the first time.

Table of contents

	Page
List of figures	7
List of supplementary movies	12
List of tables	13
Abbreviations	14
<u>Chapter 1: Introduction</u>	17
1.1 Cancer metastasis	17
1.2 The Cytoskeleton	19
1.2.1 Actin and myosin	19
1.2.2 Microtubules	23
1.2.3 Intermediate filaments	25
1.3 Cell:substrate adhesion	26
1.4 Phopholipid asymmetry in cell polarity and migration	27
1.5 Cell:cell junctions	30
1.5.1 Adherens Junctions	30
1.5.2 Tight junctions	31
1.6 The Rho GTPases	32
1.6.1 Rho GTPase regulation	33
1.6.2 Roles of Rho GTPases in cell migration	34
1.7 p21-activated kinases	39
1.7.1 PAK regulation	42
1.7.1.1 Regulation of Group I PAKs	42
1.7.1.2 Regulation of Group II PAKs	45
1.7.2 PAK expression	46
1.7.3 Roles of PAK in cell migration	47
1.7.4 Roles of PAKs in MAPK signalling	53
1.8 Problems associated with dominant negative mutants	54
1.9 RNA interference (RNAi)	55
1.10 Problems with RNAi	56
1.11 Hepatocyte growth factor signalling	58
1.12 Aims	59

<u>Chapter 2: Materials and methods</u>	61
2.1 Materials	61
2.1.1 Tissue culture materials	61
2.1.2 Protein biochemistry materials	61
2.1.3 Cell biology materials	64
2.2 Methods	68
2.2.1 Tissue culture	68
2.2.1.1 Passaging cell lines	68
2.2.2 Protein Biochemistry	68
2.2.2.1 HGF stimulation for cell lysis	68
2.2.2.2 Cell lysis	68
2.2.2.3 BioRad protein concentration assay	69
2.2.2.4 Immunoprecipitation	69
2.2.2.5 NuPAGE (SDS-PAGE using pre-cast gels)	69
2.2.2.6 SDS-PAGE using non-precast gels	70
2.2.2.7 Western blotting	70
2.2.2.8 Quantification of western blots	71
2.2.3 Cell Biology	71
2.2.3.1 Transfection of cells with siRNA using Lipofectamine 2000	71
2.2.3.2 Scattering and random migration assays	71
2.2.3.3 Analysis of cell rounding	72
2.2.3.4 Analysis of lamellipodium length	73
2.2.3.5 DU145 cell scratch assays using the Cellomics ArrayScan instrument	73
2.2.3.6 DU145 cell scratch assays using time-lapse microscopy	74
2.2.3.7 DU145 Matrigel invasion assays	74
2.2.3.8 Acid washing coverslips	75
2.2.3.9 Immunofluorescent staining	75
2.2.3.10 Confocal microscopy	76
<u>Chapter 3: PAK expression and regulation in cancer cell migration</u>	77
3.1 Introduction	77
3.2 PAK expression in cancer cell lines	78

3.3	DU145, PC3, MCF7 and HT29 cells respond to HGF	80
3.4	PAK1 and PAK2 are phosphorylated in response to HGF	81
3.5	Individual PAK isoforms can be knocked down in cancer cell lines	87
3.6	Effect of PAK knockdown on phosphorylation of PAK1 and PAK2 at T423	90
3.7	Knockdown of PAK2 leads to upregulation of PAK1	93
3.8	Cdc42 and Rac1 knockdown impair phosphorylation of S199/204 or S192/194 in PAK1 and PAK2.	97
3.9	Cdc42 and Rac1 impair PAK phosphorylation downstream of HGF	98
3.1	Discussion	108
<u>Chapter 4: Role of PAK in HGF-induced migration and invasion</u>		114
4.1	Introduction	114
4.2	The response of cancer cells to HGF	115
4.3	Effect of PAK knockdown on DU145 cell scattering	119
4.4	Effect of Rho GTPase knockdown on DU145 cell scattering	133
4.5	Phenotypes of cells following combined knockdown of PAK1 and PAK2	135
4.6	PAK1 or PAK2 knockdown impairs migration of PC3 cells	138
4.7	HGF increases migration of DU145 in wound healing assays	143
4.8	PAK knockdown does not slow DU145 cell wound healing	145
4.9	PAK1 or PAK2 knockdown does not inhibit DU145 cell invasion through Matrigel	148
4.10	Discussion	150
<u>Chapter 5: The role of PAK in HGF-induced cell signalling</u>		156
5.1	Introduction	156
5.2	PAK1 knockdown does not affect E-cadherin expression in DU145 cells following HGF stimulation	158
5.3	PAK1 or PAK2 knockdown does not affect phosphorylation of FAK downstream of HGF	159

5.4	Broad lamellipodia in PAK2-deficient DU145 cells have enriched ERK1 and GIT1/2	162
5.5	Knockdown of PAK2 impairs phosphorylation of Op18 at Serine 16 in PC3 cells but not in DU145 cells	167
5.6	Knockdown of Rac1 or Cdc42 decreases Op18 phosphorylation following HGF stimulation	170
5.7	Effect of PAK2 knockdown on microtubules in PC3 cells	170
5.8	The effect of knockdown of PAK1 or PAK2 on MLC phosphorylation	178
5.9	Knockdown of PAK1 or PAK2 does not affect phosphorylation of Raf, MEK1, ERK or p38 MAPK following HGF stimulation	181
5.10	Knockdown of PAK1 or PAK2 does not affect Akt phosphorylation in DU145 or PC3 cells	186
5.11	Discussion	189
<u>Chapter 6: Concluding remarks</u>		194
References		204
Acknowledgements		230

List of figures

		Page
Figure 1.1	Mechanisms of ECM invasion by single cancer cells	18
Figure 1.2	Actin-based structures at the leading edge of cells	20
Figure 1.3	Schematic of non-muscle myosin II	23
Figure 1.4	Polarity of the cytoskeleton in mesenchymal migration	29
Figure 1.5	Phylogenetic tree of mammalian Ras superfamily GTPases	32
Figure 1.6	Mechanism of Rho GTPase regulation	33
Figure 1.7	PAK orthologues	40
Figure 1.8	Schematic showing the conserved features of mammalian PAKs	41
Figure 1.9	Mechanism of PAK activation	43
Figure 1.10	Model for the control of Rac1, Cdc42 and PAK1 activation by	50

GIT/PIX

Figure 1.11	Control of the cytoskeleton by PAKs	53
Figure 1.12	Schematic showing wild type, constitutively active and dominant negative forms of PAK1	54
Figure 1.13	The miRNA/siRNA pathway	56
Figure 1.14	Schematic diagram of c-Met	58
Figure 2.1	Processing of images using Adobe Photoshop CS3 for cell rounding measurements	73
Figure 3.1	Morphology of DU145, PC3, MCF7 and HT29 cells	79
Figure 3.2	Cancer cell lines express PAK1, PAK2 and PAK4	80
Figure 3.3	HGF induces ERK phosphorylation in the cancer cell lines tested	81
Figure 3.4	Detection of PAK phosphorylation using the pT423 antibody	82
Figure 3.5	Effect of HGF on bands detected by an alternative phosphor-T423 antibody (Abcam)	84
Figure 3.6	HGF induces phosphorylation of PAK1 and PAK2 at S199/204	85
Figure 3.7	An antibody against phosphorylated PAK4 did not detect a change downstream of HGF	86
Figure 3.8	Titration of PAK1 siRNA in DU145 cells	88
Figure 3.9	Knockdown of individual isoforms of PAK in cancer cells	89
Figure 3.10	Effect of PAK1 knockdown on bands detected by the T423 antibody (Abcam)	91
Figure 3.11	Effect of PAK2 knockdown on bands detected by the T423 antibody (Abcam)	92
Figure 3.12	PAK1 knockdown does not affect PAK2 phosphorylation at S199/204	94
Figure 3.13	PAK2 knockdown leads to increased PAK1 phosphorylation at S199/204	95
Figure 3.14	Quantification of the effect of PAK2 knockdown on PAK1 phosphorylation at S199/204	96
Figure 3.15	Cdc42 and Rac1 affect activity of PAK1 and PAK2	97
Figure 3.16	Effect of Rac1 knockdown on bands detected by the T423 antibody (Abcam)	99

Figure 3.17	Effect of Cdc42 knockdown on bands detected by the T423 antibody (Abcam)HGF	100
Figure 3.18	Knockdown of Rac1 reduces phosphorylation of PAK1 and PAK2 at S199/204 downstream of HGF	102
Figure 3.19	Quantification of the effect of Rac1 knockdown on PAK1 or PAK2 phosphorylation at S199/204 following HGF stimulation in DU145 cells	103
Figure 3.20	Quantification of the effect of Rac1 knockdown on PAK2 phosphorylation at S199/204 following HGF stimulation in PC3 cells	104
Figure 3.21	Knockdown of Cdc42 impairs phosphorylation of PAK1 and PAK2 at S199/204 downstream of HGF	105
Figure 3.22	Quantification of the effect of Cdc42 knockdown on PAK1 or PAK2 phosphorylation at S199/204 following HGF stimulation in DU145 cells	106
Figure 3.23	Quantification of the effect of Rac1 knockdown on PAK1 or PAK2 phosphorylation at S199/204 following HGF stimulation in PC3 cells	107
Figure 3.24	STK3/4 homology with PAK1/2/3 peptide used to generate pT423 antibodies	110
Figure 3.25	Hypothetical pathways for the regulation of pS199/204 on PAK1 by PAK2	112
Figure 4.1	The response of DU145, MCF7 and HT29 cells to HGF	117
Figure 4.2	Optimization of the DU145 cell scattering assay	118
Figure 4.3	PAK1 knockdown impairs scattering of DU145 cells	120
Figure 4.4	Actin filament and E-cadherin localisation in unstimulated DU145 cells	121
Figure 4.5	E-cadherin persists in junctions in PAK1-depleted DU145 cells following HGF treatment	122
Figure 4.6	PAK2-depleted DU145 cells remain more spread following HGF stimulation	124
Figure 4.7	PAK2 knockdown in DU145 cells affects lamellipodia	125

Figure 4.8	PAK2-deficient groups of DU145 cells appear less stable	126
Figure 4.9	Actin staining in lamellipodia is different in PAK2 deficient DU145 cells following HGF stimulation	127
Figure 4.10	PAK2 knockdown leads to larger lamellipodia in DU145 cells following HGF stimulation	128
Figure 4.11	: Knockdown of PAK4 in DU145 cells	130
Figure 4.12	PAK1 knockdown decreases cell rounding in response to HGF	131
Figure 4.13	PAK knockdown does not significantly affect proliferation of DU145 cells	132
Figure 4.14	Rac1 or Cdc42 knockdown give distinct phenotypes	134-135
Figure 4.15	PAK1 and PAK2 knockdown simultaneously in DU145 cells	137
Figure 4.16	PAK1 knockdown does not affect PC3 morphology	139
Figure 4.17	PAK2 depleted PC3 cells localise in groups	140
Figure 4.18	PAK4 knockdown does not detectably affect PC3 morphology	141
Figure 4.19	PAK2 affects persistence of PC3 cell migration	142
Figure 4.20	Knockdown of PAK1 or PAK2 but not PAK4 slows migration speed of PC3 cells	143
Figure 4.21	HGF increases speed of wound healing in DU145 cells	144
Figure 4.22	PAK knockdown does not affect the speed of DU145 cell wound healing	146-147
Figure 4.23	Knockdown of PAK1 or PAK2 in DU145 cells does not affect invasion through Matrigel or migration through transwells	149
Figure 5.1	PAK1 knockdown does not affect E-cadherin down-regulation following HGF stimulation	158
Figure 5.2	PAK1 knockdown does not affect phosphorylation of FAK following HGF stimulation in DU145 or PC3 cells	160
Figure 5.3	PAK2 knockdown does not affect phosphorylation of FAK following HGF stimulation in DU145 or PC3 cells	161
Figure 5.4	Effect of PAK1 knockdown on tubulin, GIT1/2 and ERK1/2 localisation in growing cells	163
Figure 5.5	Effect of PAK1 knockdown on tubulin, GIT1/2 and ERK1/2 localisation after eighteen hours of HGF stimulation	164

Figure 5.6	Effect of PAK2 knockdown on tubulin, GIT1/2 and ERK1/2 localisation in growing cells	165
Figure 5.7	Effect of PAK2 knockdown on tubulin, GIT1/2 and ERK1/2 localisation eighteen hours of HGF stimulation	166
Figure 5.8	PAK1 knockdown does not affect the phosphorylation of Op18 after HGF stimulation	168
Figure 5.9	PAK2 knockdown impairs the phosphorylation of Op18 after HGF stimulation in PC3 cells but not DU145 cells	169
Figure 5.10	Quantification of Op18 phosphorylation in PAK2-depleted PC3 cells	170
Figure 5.11	Rac1 knockdown impairs the phosphorylation of Op18 after HGF stimulation in PC3 and DU145 cells	171
Figure 5.12	Quantification of Op18 phosphorylation in Rac1-depleted DU145 and PC3 cells	172
Figure 5.13	Cdc42 knockdown impairs the phosphorylation of Op18 after HGF stimulation in PC3 and DU145 cells	173
Figure 5.14	Quantification of Op18 phosphorylation Cdc42-depleted DU145 and PC3 cells	174
Figure 5.15	Effect of PAK2 knockdown on PC3 microtubule organisation in unstimulated cells	176
Figure 5.16	Effect of PAK2 knockdown on PC3 microtubule organisation in unstimulated cells	177
Figure 5.17	PAK1 knockdown does not affect the phosphorylation of MLC downstream of HGF	179
Figure 5.18	PAK2 knockdown does not affect the phosphorylation of MLC downstream of HGF	180
Figure 5.19	The effect of PAK1 knockdown on MAPK signalling	182
Figure 5.20	The effect of PAK2 knockdown on MAPK signalling	183
Figure 5.21	The effect of PAK1 knockdown on the phosphorylation of MEK1 downstream of HGF	184
Figure 5.22	The effect of PAK2 knockdown on the phosphorylation of MEK1 downstream of HGF	185

Figure 5.23	PAK1 knockdown does not affect Akt phosphorylation following HGF stimulation	187
Figure 5.24	PAK1 knockdown does not affect Akt phosphorylation following HGF stimulation	188
Figure 6.1	Sequence alignment of PAK1 and PAK2 surrounding the S199/204 autophosphorylation sites	197
Figure 6.2	Hypothetical model for the control of lamellipodium formation by PAK1/2 in DU145 cells	198

List of supplementary movies

Movie	Cell and treatment	Figure
Movie 1	DU145 + 10 ng/ml HGF	4.3
Movie 2	DU145 siControl + 10 ng/ml HGF	4.3, 4.6, 4.11
Movie 3	DU145 siPAK1a + 10 ng/ml HGF	4.3
Movie 4	DU145 siPAK1b + 10 ng/ml HGF	
Movie 5	DU145 siPAK1c + 10 ng/ml HGF	
Movie 6	DU145 siPAK1d + 10 ng/ml HGF	
Movie 7	DU145 siPAK2a + 10 ng/ml HGF	4.6
Movie 8	DU145 siPAK2b + 10 ng/ml HGF	
Movie 9	DU145 siPAK2c + 10 ng/ml HGF	
Movie 10	DU145 siPAK2d + 10 ng/ml HGF	
Movie 11	DU145 siPAK4a + 10 ng/ml HGF	4.11
Movie 12	DU145 siPAK4b + 10 ng/ml HGF	
Movie 13	DU145 siPAK4c + 10 ng/ml HGF	
Movie 14	DU145 siPAK4d + 10 ng/ml HGF	
Movie 15	PC3 + 10 ng/ml HGF	4.17
Movie 16	PC3 siControl + 10 ng/ml HGF	4.17, 4.18, 4.19
Movie 17	PC3 siPAK1a + 10 ng/ml HGF	4.17
Movie 18	PC3 siPAK1b + 10 ng/ml HGF	
Movie 19	PC3 siPAK1c + 10 ng/ml HGF	
Movie 20	PC3 siPAK1d + 10 ng/ml HGF	

Movie 21	PC3 siPAK2a + 10 ng/ml HGF	4.18
Movie 22	PC3 siPAK2b + 10 ng/ml HGF	
Movie 23	PC3 siPAK2c + 10 ng/ml HGF	
Movie 24	PC3 siPAK2d + 10 ng/ml HGF	
Movie 25	PC3 siPAK4a + 10 ng/ml HGF	4.19
Movie 26	PC3 siPAK4b + 10 ng/ml HGF	
Movie 27	PC3 siPAK4c + 10 ng/ml HGF	
Movie 28	PC3 siPAK4d + 10 ng/ml HGF	

List of tables

Table 1.1	Major targets of Rho GTPases involved in cell migration	35
Table 1.2	Rho GTPases shown to bind mammalian PAK isoforms	44
Table 1.3	Phenotypes of PAK knockout mice	47
Table 2.1	Reagents used for tissue culture	61
Table 2.2	Composition of solutions used for protein biochemistry	61
Table 2.3	Reagents used for protein biochemistry	62
Table 2.4	Equipment used for protein biochemistry	63
Table 2.5	Primary and secondary antibodies used for probing western blots	63
Table 2.6	Reagents used in cell biology experiments	64
Table 2.7	siRNA oligomers used for protein knockdown	64
Table 2.8	Antibodies used for immunofluorescence	66
Table 2.9	Software used for image capture, processing and analysis	67
Table 2.10	Microscopes and instruments used for image capture	67
Table 2.11	Composition of growth media used for tissue culture and experiments	67
Table 2.12	Lasers and filters used for confocal microscopy	76
Table 4.1	Observed effects of PAK isoform knockdown in DU145 and PC3 cells	155

List of Abbreviations

ADF	actin depolymerising factor
Arp2/3	actin-related protein 2/3
ARPC	actin-related protein complex
ATP	adenosine triphosphate
CA	constitutively active
CRIB	Cdc42/Rac interactive binding
DMEM	Dubecco's modified eagle medium
DN	dominant negative
dsRNA	double stranded RNA
ECM	extracellular matrix
Ena/VASP	(Drosophila) enabled/vasodilator-stimulator protein
ERK	extracellular signal-related kinase
FAK	focal adhesion kinase
FCS	foetal calf serum
FRL	Formin related protein
GAP	GTPase activating protein
GDI	guanine nucleotide dissociation inhibitor
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GIT	G protein-coupled receptor kinase interactor
GTP	guanosine triphosphate
GTPase	guanosine triphosphatase
HGF	hepatocyte growth factor
IQGAP	IQ motif-containing GTPase activating protein
IRSp53	insulin receptor substrate of 53 kDa
JAM	junctional adhesion molecule
KGF	keratinocyte growth factor
LIMK	LIM domain kinase
MAPK	mitogen activated protein kinase
MDCK	madin darby canine kidney

mDia	mammalian homologue of Drosophila diaphanous
MEK	MAPK-ERK kinase
MHC	myosin heavy chain
miRNA	micro RNA
miRNP	miRNA-containing ribonucleoprotein complex
MLC	myosin light chain
MLC	myosin light chain
MLCK	myosin light chain kinase
MMPs	matrix metalloproteinases
MOPS	3-(N-morpholino)propane sulphonic acid
MRCK	myotonic dystrophy kinase-related Cdc42-binding kinase
MT	microtubule
MTOC	microtubule organising centre
mTORC2	Mammalian target of rapamycin complex 2
Op18	oncoprotein 18
p120ctn	p120 catenin
PAK	p21-activated kinase
PBD	p21-binding domain
PBS	phosphate-buffered saline
PH	pleckstrin homology
PI3K	phosphatidylinositol-3-kinase
PIP	phosphatidyl inositol phosphate
PIP ₃	phosphatidylinositol-3,4,5-trisphosphate
PIX	PAK-interacting exchange factor
PKA	cAMP dependent protein kinase
PKC	protein kinase C
PKL	paxillin kinase linker
PM	plasma membrane
POPX	partner of PIX
PTEN	phosphatase and tensin homologue
RISC	RNA-induced silencing complex
R-MLC	regulatory myosin light chain

RNA	ribonucleic acid
ROCK	Rho kinase
RPMI	Roswell Park Memorial Institute
SAGE	serial analysis of gene expression
SDS	sodium dodecylsulphate
SH2	Src homology domain 2
SH3	Src homology domain 3
shRNA	short hairpin RNA
siRNA	short interfering RNA
TBS	Tris-buffered Saline
TCoB	Tubulin cofactor B
TLR	Toll-like receptor
VEGFA	vascular endothelial growth factor-A
VEGFR1	vascular endothelial growth factor receptor 1
WASP	Wiscott-Aldrich syndrome protein
WAVE	WASP-family verprolin homology protein
ZO	zonula occludens

Chapter 1: Introduction**1.1: Cancer metastasis**

Cancer is a multistep disease caused by gene mutations that lead to uncontrolled cell proliferation and formation of a tumour. One of the factors leading to poor patient prognosis is cancer metastasis (Pantel et al., 2008). In this process, signalling pathways controlling cell:cell junction stability and cell migration are deregulated allowing cells to escape the primary tumour, migrate to distal sites and establish secondary tumours (reviewed in Sahai, 2007). Understanding these signalling pathways will lead to the identification of potential therapeutic targets for the disease. Single cancer cells can migrate *in vivo* by two known mechanisms termed mesenchymal or amoeboid motility (Figure 1.1). The mesenchymal model of cell migration has been extensively studied because cells moving *in vitro* on a two dimensional (2D) substrate often migrate in this manner. Cells adhere to the substrate and extend protrusions at the leading edge such as large, flat lamellipodia or thin filopodia. New adhesion structures form at the front of the cell and enzymes called matrix metalloproteinases (MMPs) are secreted to degrade the extracellular matrix (ECM) allowing the cell to move through. At the tail of the cell, adhesions disassemble and contractile force pulls the cell forwards (reviewed in Ridley, 2001). Amoeboid migration is less well characterised but it is believed that the majority of metastatic cancer cells migrate in this way. Cells migrating in an amoeboid manner are thought to form weaker cell:matrix adhesions than in mesenchymal migration and cells squeeze through the ECM rather than secreting enzymes to degrade it. Contractile force generates movement by hydrostatic pressure (reviewed in Sahai, 2005). Both modes of cell migration are facilitated by the dynamic remodelling of the cell cytoskeleton and the breakdown of cell:cell junctions.

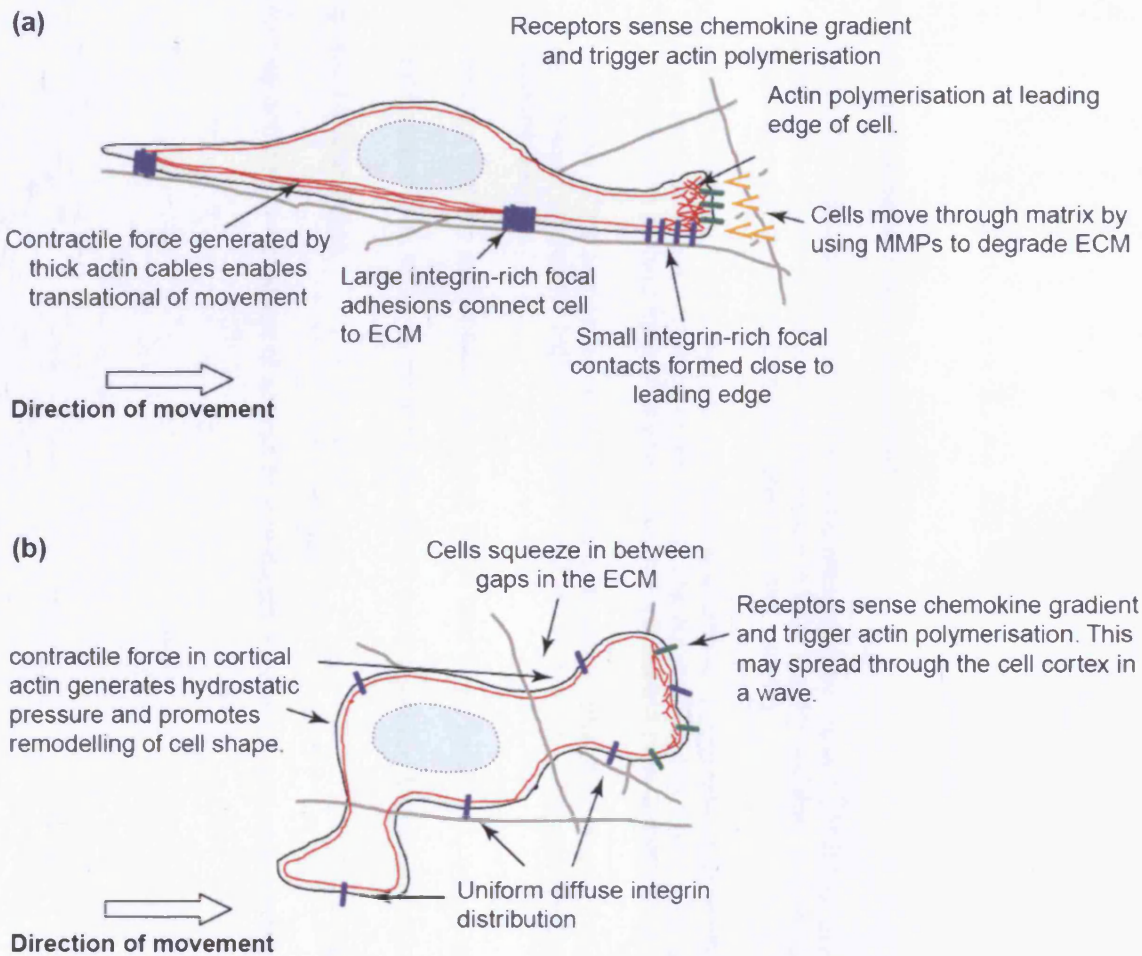


Figure 1.1: Mechanisms of ECM invasion by single cancer cells. a) In mesenchymal motility cells form strong integrin-based adhesions and migrate by protruding at the front and degrading the ECM, while contraction of actin pulls the tail forward. Chemoreceptors at the front of the cell trigger actin polymerisation and facilitate the establishment of cell polarity. b) In amoeboid migration adhesion is weak and actin contraction generates hydrostatic pressure to provide a driving force to remodel cell morphology. Receptors at the front of the cell sense the chemokine gradient to establish cell polarity. Taken from (Sahai, 2005)

1.2: The Cytoskeleton

1.2.1 Actin and myosin

The actin monomer (G-actin) is a protein with a molecular weight of around 43 kDa. It contains a nucleotide binding site which binds ATP in a complex with Mg^{2+} . Nucleotide binding serves to stabilise the actin monomer promoting polymerisation of filaments (F-actin) while subsequent adenosine triphosphate (ATP) hydrolysis and dissociation of the 3'-phosphate from F-actin functions as an indicator of the age of the age of a filament (De La Cruz et al., 2000). Actin monomers polymerise in a right handed helix with 'barbed' and 'pointed' ends. Both ends of the filament can accept and release actin monomers but filament assembly is favoured at the barbed end which generally points towards the plasma membrane.

In order for a new actin filament to polymerise, an initial actin nucleus must be formed which can then accept actin monomers. Actin dimers are unstable so this process is facilitated by actin nucleators four types of which are known in mammalian non-muscle cells: The actin-related protein 2/3 (Arp2/3) complex, comprising Arp2 and Arp3 monomers together with five actin-related protein complex (ARPC) monomers, binds to an existing filament and is thought to mimic a new barbed end promoting a branched network of F-actin (reviewed in Goley and Welch, 2006); formins, which are a family of proteins that nucleate actin and remain bound to the barbed end of the extending filament (reviewed in Kovar, 2006); cordon-bleu, a newly identified actin nucleator that controls neuronal morphology ((Ahuja et al., 2007) and reviewed in Winckler and Schafer, 2007); and Spire, which was originally identified in *Drosophila* but is also found in mammals (reviewed in Kerkhoff, 2006). In muscle cells a further actin nucleator, Leiomodin has also recently been identified (Chereau et al., 2008).

Filament assembly, disassembly, branching and bundling are controlled by many actin binding proteins and much research is devoted to uncovering how they bring about the multitude of actin-based structures found in cells. Examples of these structures in motile cells are lamellipodia in the leading edge which contain a branched network of F-actin and filopodia which contain bundled F-actin in a finger-like protrusion (Figure 1.2).

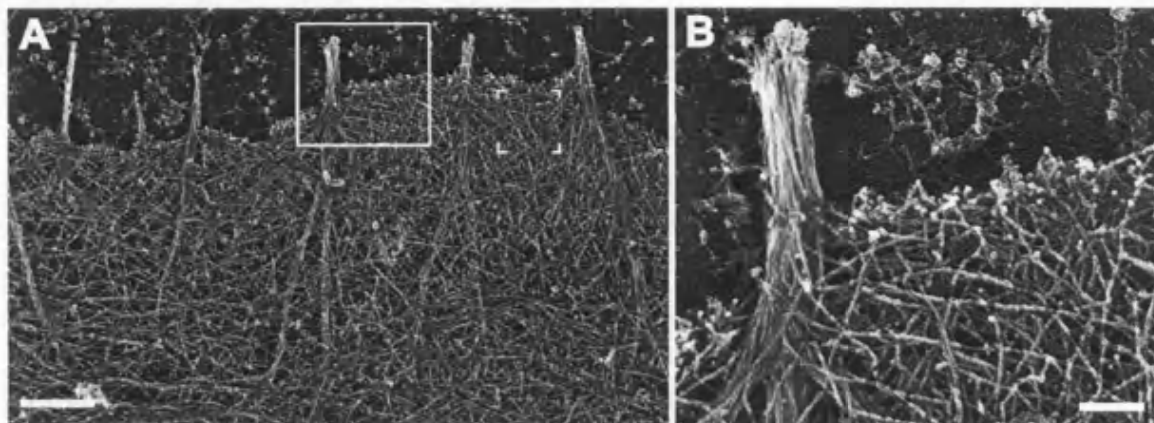


Figure 1.2: Actin-based structures at the leading edge of cells. A) A scanning electron micrograph of a lamellipodium showing the actin network. B) Increased size image of bundled actin in a filopodium (Adapted from (Yang et al., 2007)).

The regulation underlying the generation of lamellipodia and filopodia is poorly understood. It is thought that Arp2/3 plays an important role in branching and nucleation in lamellipodia under the regulation of its activator WASP-family verprolin homology protein (WAVE) (Lai et al., 2008; Sarmiento et al., 2008; and reviewed in Le Clainche and Carlier, 2008). However, Arp2/3 also plays a role in filopodium formation so the architecture of actin filaments at the leading edge is likely to be governed by a balance in the recruitment and activity of Arp2/3 and other proteins such as actin cross-linkers and barbed end capping proteins. For example, the actin cross-linker filamin-A has been shown to be required for the formation of dynamic membrane protrusions or membrane ruffles by p21-activated kinase 1 (PAK1) (Vadlamudi et al., 2002) and profilin, an actin binding protein that promotes monomer addition to barbed ends, can also play a role in lamellipodia formation (Kang et al., 1999; Syriani et al., 2008). This is further controlled by Ena/VASP family proteins which are thought to stimulate filament elongation, and protect barbed ends from capping proteins that would otherwise block elongation (Barzik et al., 2005; Bear et al., 2002). However, a study in *Dictyostelium* suggested that VASP does not compete with capping proteins but is instead important in the generation of filopodia via its actin-bundling activity (Schirenbeck et al., 2006). Another actin bundling protein, IQGAP serves a second function as a scaffold for signalling proteins, binding to actin, Cdc42 and N-WASP (reviewed in Brandt and Grosse 2007). IQGAP also stabilises and increases the levels of GTP-bound, active Cdc42 (see review Briggs and Sacks 2003).

In filopodia, formins such as mDia2, which nucleate actin filaments and remain bound to the barbed end leading to long, unbranched filaments play a major role in actin polymerisation. mDia2 localises to the tips of filopodia and mDia2 knockdown blocks filopodium formation. However mDia2 knockdown also impaired the formation of lamellipodia suggesting it plays a more universal role in protrusion (Yang et al., 2007). Wiscott-Aldrich syndrome protein (WASP) leads to filopodium formation upstream of the Arp2/3 complex (reviewed in Carlier et al., 1999) and it is likely that actin cross linkers such as HSP90, which was shown to bundle the filaments generated by WASP/Arp2/3, are required to produce filopodia rather than lamellipodia (Park et al., 2007).

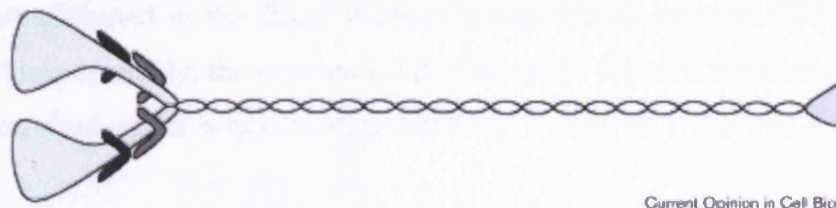
The dynamic binding and release of actin monomers is also important in membrane protrusion. In order for actin to polymerise at the membrane, the cell requires a pool of free actin monomers. The supply of monomers is controlled by cofilin which can sever F-actin filaments to produce new barbed ends (Maciver, 1998) and increase the off-rate from pointed ends to promote F-actin disassembly (Carlier et al., 1997). It has recently been shown that cofilin is required for stimulus-induced lamellipodium formation (Kiuchi et al., 2007) showing its importance in actin dynamics. It also highlights a key point that lamellipodium formation and cell migration do not only rely on actin polymerisation and filament assembly alone, but the dynamic cycling of actin from the barbed end to the pointed end and back into the G-actin pool. In fact, profilin-1 (which promotes G-actin addition to barbed ends) is down-regulated in a variety of human cancers and cancer cell lines (Gronborg et al., 2006; Janke et al., 2000; Wu et al., 2006), and knockdown of the basal level of profilin-1 in MDA-MB-231 breast cancer cells leads to increased motility and invasion through Matrigel (Zou et al., 2007).

Actin plays another role in cell migration through its role in contractile force generation. This is apparent *in vitro* on a 2D substrate where individual filaments of F-actin can be bundled together to form stress fibres, each containing around 10-30 individual actin filaments (Cramer et al., 1997). It should be noted however, that stress fibres are rarely found *in vivo*. There are three different types of actin stress fibre found in cells: transverse arcs which are not anchored to adhesions (Heath, 1983); dorsal stress fibres which are anchored to a focal adhesion at one end and ventral stress fibres anchored to focal adhesions at both ends (reviewed in Small et al., 1998). Focal adhesions are the sites of

cell:matrix adhesion found in some cell types. Three distinct mechanisms for the formation of stress fibres have been described. It is thought that dorsal stress fibres are assembled via actin polymerisation driven by formin at focal adhesions while transverse arcs are either formed by bundles of myosin and actin being joined in the lamella (Hotulainen and Lappalainen, 2006) or by the effect of myosin contraction on actin networks drawing actin filaments together (Verkhovsky et al., 1995).

The polarity of actin filaments in stress fibres appears to be different depending on the situation. It has been described that actin filaments can either arrange with uniform polarity or with seemingly random orientation (Cramer et al., 1997; Svitkina et al., 1997). It is probable that the mechanism by which a stress fibre is generated could dictate the orientation of the actin filaments it contains. Actin filaments are bound together by actin cross-linking proteins of which several have been found associated with stress fibres such as fascin, espin, filamin and α -actinin (Adams, 1995; Chen et al., 1999; Lazarides and Burridge, 1975; Wang et al., 1975). Different actin cross-linkers are thought to cooperate to modulate the mechanical properties of the actin cytoskeleton (Tseng et al., 2005). Immunostaining of actin bundles revealed that α -actinin and filamin-A are found in bands along the length of stress fibres with bands of myosin between (Langanger et al., 1984; Langanger et al., 1986).

Myosins are a superfamily of at least 15 classes of molecular motor proteins in mammals (reviewed in Sellers, 2000). The non-muscle myosin II class is involved in contraction and shortening of stress fibres in non-muscle cells. Much of the research investigating Myosin II has been done in relation to smooth muscle contraction but it is thought that the mechanism is similar in non-muscle cells. Myosin II is a hexamer composed of two heavy chains, two essential light chains and two regulatory light chains (RLC) (Figure 1.3). Multiple myosin II hexamers associate to form bipolar filaments which bind actin at each end and hydrolyse ATP to pull the two actin filaments together. Myosin II filament assembly, ATPase activity and contraction are regulated by phosphorylation of MHC and RLC at multiple sites (reviewed in Tan et al., 1992). Specifically, phosphorylation of RLC at Threonine 18 and Serine 19 is thought to lead to myosin filament assembly and increased contractility (Watanabe et al., 2007).



Current Opinion in Cell Biology

Figure 1.3: Schematic of non-muscle myosin II. Showing heavy chains (light grey), essential light chains (dark grey) and regulatory light chains (black). (Taken from Bresnick 1999).

Myosin IIA was shown to be required for tail retraction in migrating cells (Even-Ram et al., 2007). The mechanism for this could be by the action of myosin II on ventral stress fibres physically pulling the tail forwards combined with the stimulation of focal adhesion disassembly by other pathways.

Actin also plays a major role in polarised cell locomotion (Figure 1.4). In order for a cell to migrate persistently it needs to establish an intracellular polarity reflected by features of the actin cytoskeleton and microtubule network. Actin polymerisation is localized towards the leading edge of the cell where actin nucleation and branching are stimulated leading to the generation of an actin network of filaments growing towards the membrane (Henson et al., 1999; Small et al., 1978). Retrograde flow of the actin cytoskeleton is observed (Henson et al., 1999) and the nature of the actin network changes in a gradient from dense, highly branched filaments at the front to sparse bundles of aligned filaments at the rear (Svitkina et al., 1997). Towards the rear of the cell, increased contractility driven by myosin either pulls the tail forwards or the sides of the cell inwards. Evidence supports a model where a gradient of myosin incorporation into actin bundles could lead to the change in the morphology of the actin network and increased contraction at the rear of the cell. (Svitkina et al., 1997; Verkhovsky et al., 1995, 1997). This is supported by evidence of a gradient of contractility towards the rear of the cell driving cell components forwards (Iwasaki and Wang, 2008).

1.2.2 Microtubules

Microtubules (MTs) are cylindrical tubes composed of 13 parallel protofilaments. These protofilaments are formed by the polymerisation of heterodimers of α and β tubulin. Both monomers can bind GTP. α -tubulin contains an N-terminal GTP-binding site that is non-

exchangeable and buried in the dimer while β -tubulin has an exposed GTP-binding site. Upon microtubule assembly, the exposed GTP-binding site contacts the newly added dimer where catalytic residues on α -tubulin stimulate GTP hydrolysis (reviewed in Nogales and Wang, 2006).

Like actin filaments, MTs have distinct ends; the plus-end is usually directed towards the periphery of the cell while the minus end is usually anchored to the microtubule organising centre (MTOC) which is the site of MT nucleation. γ Tubulin is involved in MT nucleation but the mechanism for this is unclear. Evidence in yeast suggests that γ Tubulin may also play a role in the regulation of MT plus ends but what this role is remains to be shown (Raynaud-Messina and Merdes, 2007; Watanabe et al., 2005). Microtubule assembly and disassembly can be controlled by microtubule destabilisation proteins such as stathmin/Op18, which can either sequester tubulin dimers or promote microtubule disassembly at the plus end (Belmont and Mitchison, 1996; Howell et al., 1999; Jourdain et al., 1997; Larsson et al., 1999). Op18 is inhibited by phosphorylation on one or more of four serine residues (Ser16, Ser25, Ser38, Ser63). Multiple kinases have been identified that can phosphorylate these sites (reviewed in Cassimeris 2002).

One of the functions of MTs is to serve as an intracellular track along which motor proteins of the dynein and kinesin families can move. Dynein and kinesin carry vesicles along MTs in a directional manner; dyneins move towards the minus end while most kinesins move towards the plus end (reviewed in Caviston and Holzbaur, 2006). The microtubule network polarises in motile cells (Figure 1.4). In many cell types the centrosome is reorientated towards the direction of migration and microtubules become orientated towards the leading edge (Etienne-Manneville and Hall, 2001; Euteneuer and Schliwa, 1992; Gomes et al., 2005; Gotlieb et al., 1981; Gregory et al., 1988; Gundersen and Bulinski, 1988; Kupfer et al., 1982).

Most of these studies were carried out using *in vitro* wound healing but it is thought that microtubule polarity also occurs in migration of single cells (reviewed in Wittmann and Waterman-Storer, 2001). Microtubule growth in migrating cells is promoted at the leading edge to prevent plus ends moving backwards with retrograde flow of actin filaments (Wadsworth, 1999; Waterman-Storer and Salmon, 1997). The roles of microtubules in cell

polarity are probably mediated through their facilitation of intracellular transport by motor proteins such as kinesin and dynein. Membrane and associated proteins including recycled receptors are transported to the leading edge during migration and transmigration and this is thought to depend on the microtubule network (Bergmann et al., 1983; Hopkins et al., 1994; Mamdouh et al., 2008). A possible pathway for the stabilisation of microtubules in cell migration is through p21-activated kinase 1 (PAK1) which phosphorylates Op18 at Ser16 inhibiting it (Wittmann et al., 2004). PAK1 will be described in detail later.

It has also been suggested that microtubules have a direct effect on membrane ruffling independent of their transport activity via the activation of Rac1 (Waterman-Storer et al., 1999). However, it is possible that this effect is due to the delivery of Rac1 GEFs or IQGAP to the leading edge via microtubule plus ends (reviewed in Siegrist and Doe, 2007). A further role for microtubules in focal adhesion disassembly has been identified and it is believed that this requires molecular transport by kinesin (Bershadsky et al., 1996; Kaverina et al., 1999; Kaverina et al., 1998; Krylyshkina et al., 2002).

As well as their role in migration, MTs are also responsible for the segregation of chromosomes during mitosis (reviewed in O'Connell and Khodjakov, 2007).

1.2.3 Intermediate filaments

Intermediate filaments are formed from polymers of protein subunits and extend throughout the cell tethering to organelles, other cytoskeletal components, the nucleus and the extracellular matrix via linker proteins (reviewed in Goldman et al., 2008). Many intermediate filament proteins are cell-type specific and their major roles are believed to be structural integrity and mechanotransduction (see review Herrmann et al., 2007). Intermediate filaments also play roles in cell migration and invasion. Initial investigation of the presence of intermediate filament proteins in tumours indicated that vimentin (normally expressed in mesenchymal cells) was sometimes coexpressed with keratin (an epithelial intermediate filament) in metastatic tumours (Miettinen and Rapola, 1989; Ramaekers et al., 1983; Zarbo et al., 1990). Further studies using vimentin-positive melanoma cell lines showed that expression of keratin correlates with invasive potential, and disruption of keratin in the highly metastatic C8161 cells decreased invasive and metastatic potential (Hendrix et al., 1992). Further work showed that expression of keratin 8 or 18 in the poorly

invasive, vimentin-positive A375P melanoma cell line increased their invasion and migration ability (Chu et al., 1996).

Other data show that vimentin may also play an individual role; vimentin null fibroblasts have defects in migration, chemotaxis and contraction (Eckes et al., 1998). It was later found that vimentin overexpression correlates with invasiveness of prostate cancer cells (Wei et al., 2008) and knockdown of vimentin impaired migration and collagen invasion of SW480 colon cancer and MDA-MB-231 breast cancer cell lines (McInroy and Maatta, 2007). The effect of vimentin on cell migration and invasion could be mediated through modulation of cell adhesion as vimentin depletion by RNAi leads to cells with smaller focal contacts and decreased adhesion (Tsuruta and Jones, 2003). Vimentin associates with focal adhesions through integrin binding (Kreis et al., 2005) and is believed to play a role in cell migration via integrin recycling in a process which requires vimentin phosphorylation by PKC ϵ (Ivaska et al., 2005). Vimentin phosphorylation at multiple sites by p21-activated kinase (PAK), at different sites to those phosphorylated by PKC ϵ , is also thought to control filament organization (Goto et al., 2002; Li et al., 2006).

1.3 Cell:substrate adhesion

As previously mentioned, adhesion is weak in amoeboid migration where cell surface adhesion molecules are diffuse but in mesenchymal migration cell:substrate interaction is stronger and occurs at defined regions in the cell membrane termed focal contacts or focal adhesions (Abercrombie and Dunn, 1975). The main proteins responsible for extracellular matrix (ECM) adhesion at focal complexes are the integrins. These are heterodimers containing α and β subunits, each of which has a large extracellular domain, a single transmembrane domain and a small intracellular domain. In vertebrates there are 18 known α and 8 known β subunits which form multiple combinations (reviewed in Luo et al., 2007). Inactive integrins exist in a closed conformation and can be activated in an extracellular manner by binding their ligand (outside-in) or signalling inside the cell can lead to activation (inside-out) (reviewed in Hynes, 2002). Integrins cluster at sites of adhesion and recruit many structural and cell-signalling molecules which are responsible for kinase signalling following integrin binding and physical association with the cytoskeleton. The association with actin filaments is important in cell migration as this provides a means for the cell to exert force on the substrate to pull itself forwards. Several proteins are believed

to play a role in the connection between integrins and the cytoskeleton. One of these is talin and is believed to be required for the integrin:actin connection (Cram et al., 2003; Lee et al., 2004; Tanentzapf et al., 2006). Vinculin and α -actinin also play a role, probably in complex with talin (reviewed in Ziegler et al., 2008). Pathways regulating focal adhesion stability are not fully understood but signalling through focal adhesion kinase (FAK) has been shown to induce focal adhesion disassembly and FAK knockout mice have fibroblasts with larger and more numerous focal adhesions (Ilic et al., 1995; Webb et al., 2004).

Strength of adhesion to the substrate is polarised in motile cells to allow detachment from the substrate at the rear and formation of new adhesions at the front (reviewed in Broussard et al., 2008). It is clear that a spatial balance is required between pathways driving focal adhesion assembly and disassembly in the cell as it has been suggested that increased contractility can lead to focal adhesion assembly (Chrzanowska-Wodnicka and Burridge, 1996).

1.4: Phospholipid asymmetry in cell polarity and migration

The phosphorylation state of membrane lipids is likely to be important in some cell types to establish cell polarity and stimulate protrusion (Figure 1.5). Initial studies in *Dictyostelium discoideum* showed that pleckstrin homology (PH) domain-containing proteins localized towards the source of chemoattractant (Meili et al., 1999; Parent et al., 1998). Some PH domains bind to phosphatidylinositol-3,4,5-trisphosphate (PIP_3) and $\text{PI}(3,4)\text{P}_2$, both products of phosphatidylinositide-3-kinases (PI3Ks). (For a review of the discovery of PI3K and PIP_3 see Stephens et al., 1993). PIP_3 can be dephosphorylated to $\text{PI}(4,5)\text{P}_2$ by phosphatase and tensin homologue (PTEN). It was found that PI3K and PTEN are reciprocally regulated in response to a chemotactic gradient with PI3K localizing towards the chemoattractant and PTEN localizing away from it (Funamoto et al., 2002; Iijima and Devreotes, 2002). Asymmetric phospholipid distribution in a chemotactic gradient is not limited to *Dictyostelium*; it was shown that PIP_3 -binding PH domains show a similar localization in neutrophils and fibroblasts (Haugh et al., 2000; Wang et al., 2002b). However, the importance of phospholipid distribution in cells is unclear. PI3K γ -dependent PIP_3 accumulation is not required for gradient sensing in neutrophils (Ferguson et al., 2007) so it is likely that other signalling mechanisms such as Rho GTPases and effector localization can still lead to cell polarization in the absence of PIP_3 (reviewed in Ward,

2004). Likewise, in *Dictyostelium* genetic deletion experiments showed that PI3K deficient cells could still migrate towards a cAMP gradient but this was blocked by the combined deletion of PI3K and a phospholipase A₂ homologue suggesting that the pathways act in parallel (Chen et al., 2007b).

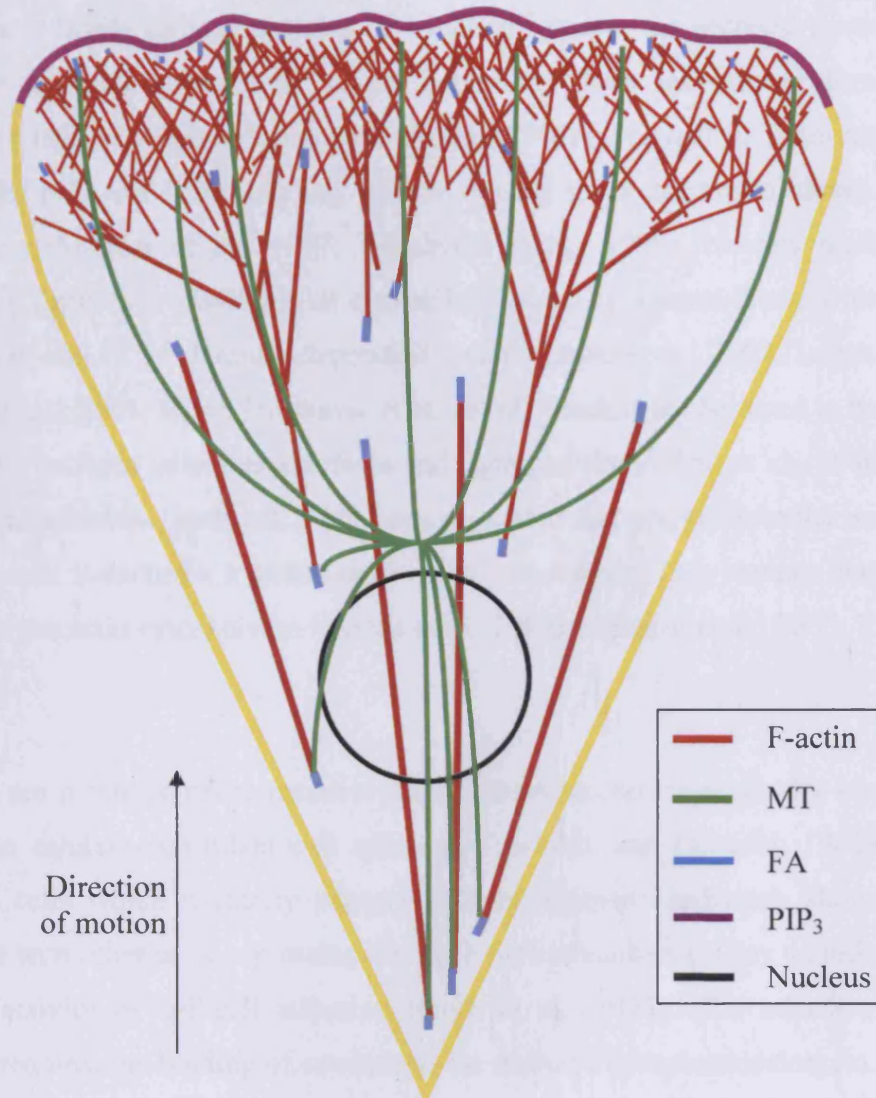


Figure 1.4: Polarity of the cytoskeleton in mesenchymal migration. As described in the text, actin polymerisation occurs at the leading edge and bundles as it moves towards the back of the cell forming stress fibres. New focal adhesions form and turnover at the front of the cell, some of which develop into larger mature focal adhesions at either end of the stress fibres. The MTOC is localized in front of the nucleus and microtubules orient towards the leading edge to facilitate protrusion, and towards focal adhesions in the rear to facilitate tail detachment. The presence of active PI3K in the leading edge is thought to increase local PIP₃ concentration in some cell types.

1.5: Cell:cell junctions

1.5.1: Adherens Junctions

Adherens junctions are formed by two classes of adhesion molecule, nectins and cadherins. Nectins are a family of four proteins of which all, except the secreted protein nectin-1 γ , have an N-terminal extracellular region containing three IgG-related domains and an intracellular region which interacts with afadin (AF-6) (reviewed in Takai and Nakanishi, 2003). Afadin is an actin binding protein which gives nectins a direct link to the cytoskeleton (Mandai et al., 1997; Takahashi et al., 1999). Nectins mediate cell:cell adhesion by forming a *cis*-dimer on one cell followed by a *trans*-dimer with nectins in a neighbouring cell in a calcium-independent manner (Aoki et al., 1997; Lopez et al., 1998; Reymond et al., 2001; Satoh-Horikawa et al., 2000). Nectins are believed to be required for the assembly of both adherens junctions and tight junctions (Sato et al., 2006; Yamada et al., 2006). In adherens junctions, it has been suggested that nectins form the initial adhesion site and recruit E-cadherin together with associated catenins in a manner that requires the presence of the actin cytoskeleton (Honda et al., 2003; Okamoto et al., 2005; Yamada et al., 2004).

Cadherins are a family of transmembrane adhesion molecules originally identified to be involved in calcium-dependent cell adhesion (Yoshida and Takeichi, 1982). Expression studies in cells which normally express little endogenous cadherins showed that cells aggregated with other cells expressing the same type of cadherin. They therefore play a role in the selectivity of cell:cell adhesion (Nose et al., 1988). The adhesive function of cadherins requires the binding of catenins to the cadherin cytoplasmic domain. beta-catenin and p120-catenin (p120) bind directly while alpha-catenin binds via beta-catenin (Ozawa et al., 1989; Ozawa et al., 1990). alpha-catenin was originally thought to link cadherin to the actin cytoskeleton (Rimm et al., 1995) but it now appears that alpha-catenin binding to beta-catenin or actin is mutually exclusive (Drees et al., 2005; Yamada et al., 2005). alpha-catenin may instead regulate the actin cytoskeleton at adherens junctions, either by binding directly to actin and blocking Arp2/3 complex-mediated nucleation or by controlling the localization of the formin Fmn1 (Drees et al., 2005; Kobiela et al., 2004; Verma et al., 2004). beta-catenin and p120 regulate cadherin adhesion and localization in pathways controlled by phosphorylation. Tyrosine phosphorylation of beta-catenin disrupts its binding to E-cadherin while tyrosine phosphorylation of p120 leads to loss of E-cadherin

from the cell surface (Rosato et al., 1998; Roura et al., 1999). There is evidence that there is a constant cycle of endocytosis of unbound cadherin in complex with beta-catenin which is then recycled to the plasma membrane (Le et al., 1999). p120 knockdown leads to targeting of cadherin for degradation (Davis et al., 2003; Xiao et al., 2003). It is therefore possible that p120 binding to cadherin could decrease cadherin turnover increasing adhesion.

1.5.2: Tight junctions

Tight junctions are required for epithelial barrier function but are also involved in cell signalling to regulate junction assembly and gene expression (reviewed in Matter and Balda, 2003). Tight junctions are formed by four types of transmembrane protein; claudins; occludin; tricellulin and junctional adhesion molecules (JAMs) (Furuse et al., 1998; Furuse et al., 1993; Martin-Padura et al., 1998; McCarthy et al., 1996). The claudin family of proteins consist of four transmembrane regions, two extracellular loops and a C-terminal intracellular domain. They are important in determining the barrier function of tight junctions (Furuse et al., 1998 and reviewed in Krause et al., 2008). Occludin, the first identified component of tight junctions, has similar features to claudins but has a second intracellular domain at the N-terminus (Furuse et al., 1993). It is thought that Occludin plays a role in signal transduction from tight junctions (reviewed in Chiba et al., 2008). Tricellulin is a four transmembrane protein found in tricellular junctions of epithelial sheets (Ikenouchi et al., 2005). It appears to be particularly important in the ear as tricellulin mutation can lead to deafness (Riazuddin et al., 2006). JAMs are a family of IgG-like adhesion molecules. As well as tight junction adhesion, JAMs are thought to be involved in many other processes including leukocyte transendothelial migration and angiogenesis (reviewed in Mandell and Parkos, 2005). Many of these tight junction proteins bind to multiple cytoplasmic scaffolds. An important group of scaffold proteins associated with tight junctions are the zona occludens (ZO) proteins ZO-1, ZO-2 and ZO-3 (Itoh et al., 1999). Of these, ZO-1 was the first identified (Stevenson et al., 1986) and has multiple binding partners including claudins, JAM-1, actin and α -catenin (Itoh et al., 1999; Itoh et al., 1997). This allows ZO-1 to provide a possible link between tight junctions and the actin cytoskeleton. It has been suggested that binding between ZO-1 and α -catenin at adherens junctions could play a role in the formation of tight junctions: characterisation of E-cadherin knockout mice showed that epidermal barrier function was impaired, as was the localization of ZO-1, claudin-1 and claudin-4 to junctions. However, occludin localization

was not affected showing that incomplete tight junctions still formed (Tunggal et al., 2005). The role of E-cadherin in tight junction formation is therefore still unclear.

1.6: The Rho GTPases

Cytoskeletal dynamics during cell migration are controlled by Rho GTPases. This family of small GTPases is part of the Ras superfamily of GTP binding proteins which also includes the Rab, Arf and Ran families. There are twenty identified members of the Rho GTPase family which can be placed in 8 subgroups (Figure 1.5).

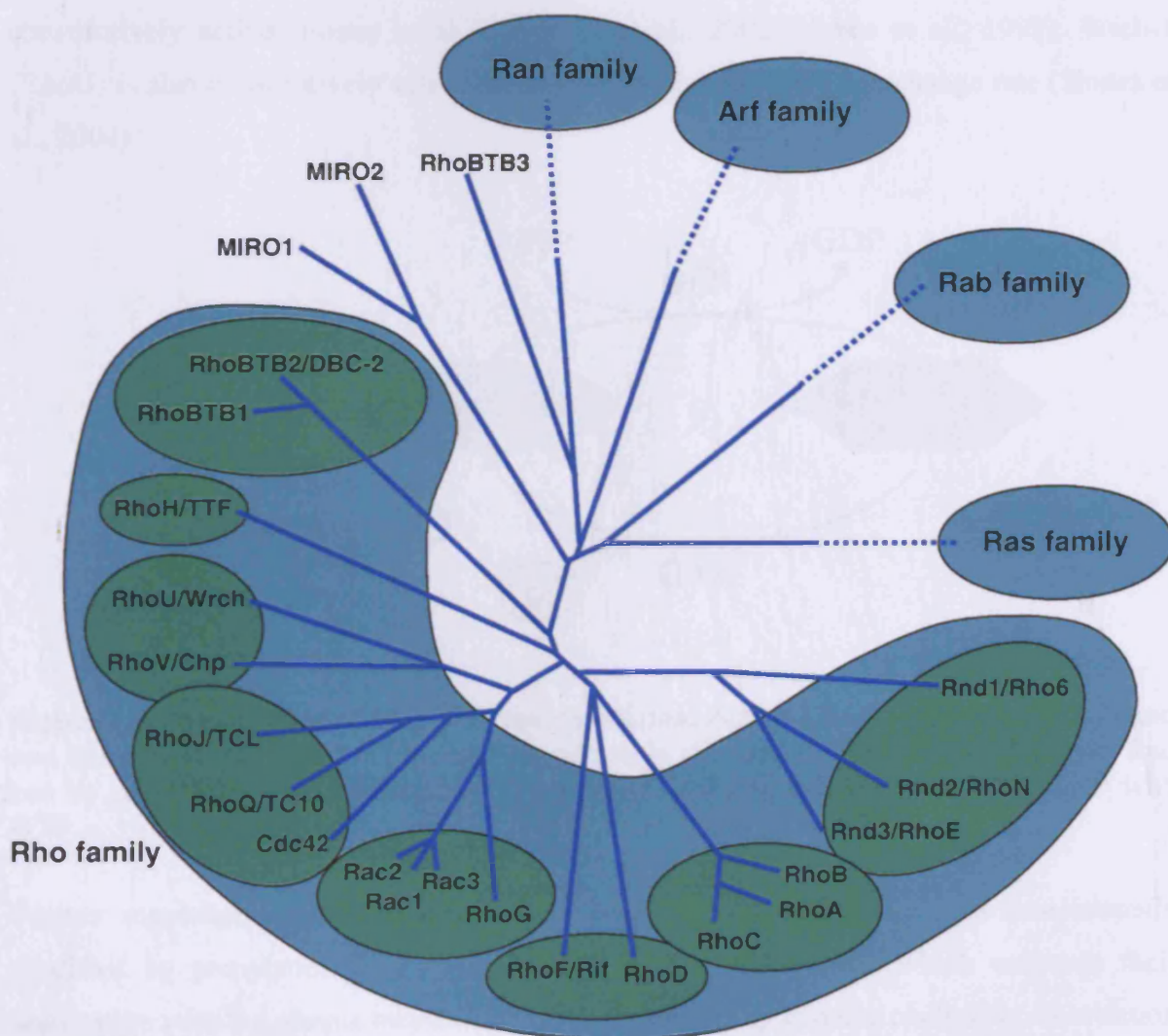


Figure 1.5: Phylogenetic tree of mammalian Ras superfamily GTPases. (Taken from (Vega and Ridley, 2008))

1.6.1: Rho GTPase regulation

Most Rho GTPases bind GTP and GDP with a high affinity and therefore usually exist in a nucleotide-bound state *in vivo* (Li and Zhang, 2004). Many of the Rho GTPases cycle from an active, GTP-bound state to an inactive, GDP-bound state. This is catalysed by two classes of regulators; GTPase activating proteins (GAPs) that stimulate hydrolysis of bound GTP to GDP; and guanine nucleotide exchange factors (GEFs) which catalyse exchange of GDP for GTP (Figure 1.5). GTP-bound to Rho GTPases are able to bind effectors and stimulate downstream signalling. Some of the Rho GTPases including Rnd proteins and RhoH have no detectable GTPase activity and it is thought that they are therefore constitutively active (Foster et al., 1996; Li et al., 2002; Nobes et al., 1998). Wrch-1 (RhoU) is also constitutively active because of its high GDP/GTP exchange rate (Shutes et al., 2004).

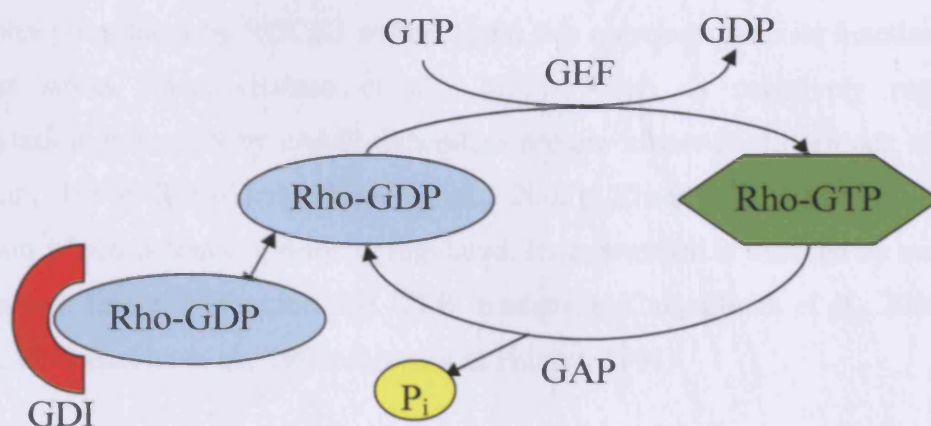


Figure 1.6: Mechanism of Rho GTPase regulation. Active Rho GTPases are GTP-bound and many have intrinsic GTPase activity which is stimulated by GAPs. GDP-bound Rho can be sequestered and inhibited by GDIs while GEFs stimulate exchange of GDP with GTP.

Further regulation occurs by localization. Many Rho GTPases are post-translationally modified by prenylation at a CAAX motif in their C-terminus, which enhances their association with the plasma membrane. This is regulated by guanine nucleotide dissociation inhibitors (GDIs) which block membrane localization by masking the prenyl group. Not all Rho GTPases are regulated in this way for example RhoBTB1 and RhoBTB2 lack the CAAX motif for prenylation (Gosser et al., 1997; Longenecker et al., 1999) (Figure 1.6). It has been described that RhoGDIs can interact with the GTP-bound form of Rac1 and RhoA

blocking GTPase activity. The association with GTP-bound protein is 10-fold lower than with GDP-bound Rac1/RhoA and the relevance *in vivo* is unknown (Sasaki et al., 1993). Regulation by GDIs is not universal among the Rho GTPases and some, such as TC10 (RhoJ) probably do not bind to GDIs (reviewed in (DerMardirossian and Bokoch, 2005)).

GEF and GAP regulation is of clear importance to the correct functioning of Rho GTPases and deregulation of some GAPs have been implicated in disease. Each family contains over 60 proteins with differing GTPase specificity. This allows each Rho GTPase to be switched on or off selectively according to the stimulus or cell state. GAPs, GEFs and GDIs can be regulated by protein-protein interaction; phosphorylation; lipid interaction; cell localisation and proteolytic degradation (reviewed in (Bernards and Settleman, 2004)).

RhoGTPases can also be regulated by phosphorylation or by transcriptional regulation. RhoE is phosphorylated by ROCK1 at Ser11 and this correlates with its functional activity to disrupt stress fibres (Riento et al., 2005). RhoA is negatively regulated by phosphorylation at Ser188 by cAMP-dependent protein kinase A (Ellerbroek et al., 2003; Lang et al., 1996; Rolli-Derkinderen et al., 2005). RhoB is a negative regulator of proliferation which is transcriptionally regulated. Its expression is induced by many factors such as growth factor stimulation and UVB irradiation (Canguilhem et al., 2005; Chen et al., 2007a; de Cremoux et al., 1994; Jahner and Hunter, 1991)

1.6.2: Roles of Rho GTPases in cell migration

Early characterisation of three of the Rho GTPases showed that they have distinct effects on the actin cytoskeleton. Microinjection of cells with active RhoA led to formation of actin stress fibres (Ridley and Hall, 1992); Rac1 led to the formation of membrane ruffles and lamellipodia (Ridley et al., 1992); while Cdc42 stimulated formation of filopodia (Nobes and Hall, 1995). This implied that Rho GTPases could be key regulators of cell migration and further research has shown this to be the case. There is also evidence that many Rho GTPases are upregulated at the mRNA or protein level in human cancers and it is thought that they play a part in tumour progression (see review (Lozano et al., 2003)). Rho GTPases take part in each feature of cell migration; cell polarisation and directional sensing; junctional breakdown; protrusion of lamellipodia; formation of new adhesions; cell

body contraction and adhesion breakdown in the tail. Some of the major downstream targets of RhoA, Rac1 and Cdc42 associated with cell migration are shown (Table 1.1).

Rho GTPase	Target	Cellular effects	References/Reviews
RhoA	ROCK	Contractility regulation and stress-fibre formation.	(Pellegrin and Mellor, 2007)
	mDia	Actin nucleation and filament assembly	(Goode and Eck, 2007)
Rac1/Cdc42	PAKs	Actin polymerisation, microtubule stability, contractility	(Bokoch, 2003)
	mDia	Actin polymerisation	(Goode and Eck, 2007)
	IRSp53	Filopodia and lamellipodia formation	(Abou-Kheir et al., 2008; Lim et al., 2008)
	IQGAP	Actin cross linking and assembly	(Brandt and Grosse, 2007)
	Sra-1(WAVE)	Lamellipodia formation and actin polymerisation	(Takenawa and Suetsugu, 2007)
	WASP	Filopodia formation and actin polymerisation	(Takenawa and Suetsugu, 2007)

Table 1.1: Major targets of RhoA, Rac1 and Cdc42 involved in cell migration.

i) Cell junction breakdown

RhoA, Rac1 and Cdc42 are important for the formation of cadherin-based cell junctions (reviewed in Braga, 2000). Interestingly RhoA, Rac1 and Rac3 have also been shown to play roles in epithelial junction breakdown suggesting that up-regulation of their expression or activity could be relevant to the first stages of cancer metastasis (Sahai and Marshall, 2002; Akhtar and Hotchin, 2001; Braga et al., 2000; Lozano et al., 2008). Investigation of the dual role of RhoA suggested that RhoA is required for adherens junction stability through mDia1 whereas RhoA-associated kinase (ROCK) activation downstream of RhoA

leads to junctional breakdown (Sahai and Marshall, 2002). The latter effect was confirmed with siRNA which showed that a RhoA/ROCK II pathway is required for epithelial junctional breakdown stimulated by removal of Ca^{2+} (Samarin et al., 2007). In the case of Rac, active Rac1 and Rac3 were shown to induce disassembly of cell junctions and Rac1-mediated junctional breakdown requires the presence of PAK1 (Akhtar and Hotchin, 2001; Braga et al., 2000; Lozano et al., 2008; Sahai and Marshall, 2002). Little is known about whether Cdc42 can facilitate junctional breakdown but as it activates PAK1, it might be expected that constitutively active Cdc42 would have a similar effect to Rac1.

ii) Cell polarity

Expression of dominant negative Cdc42 in neutrophils leads to cells that have multiple leading edges (Srinivasan et al., 2003). This blocks directional chemotaxis even though the cells can still migrate (Allen et al., 1998; Srinivasan et al., 2003). Cdc42 is also required for orientation of the Golgi apparatus and polarity of the leading edge in fibroblast scratch wound healing assays (Nobes and Hall, 1999). A model has been proposed where activated G $\beta\gamma$ binds PAK1 associated with the Cdc42-GEF α -PIX. This would lead to localized Cdc42 activation, which in turn would activate PAK leading to cytoskeletal rearrangement and generation of a leading edge (Li et al., 2003). It is likely that there is positive feedback in this model as overexpressed PAK1 which became phosphorylated at S199/S204 (a site which inhibits PIX binding), localised to membrane ruffles, and this was blocked by overexpression of the WASP-CRIB to inhibit Cdc42 (Cau and Hall, 2005). Fluorescence lifetime imaging microscopy (FLIM) has provided further evidence for the spatial relationship between PAK1 and Cdc42 as they were shown to interact at the plasma membrane, particularly in membrane protrusions (Parsons et al., 2005). Although these findings implicate a controlling role for Cdc42 in cell polarity, it is likely that other pathways are also required. Fibroblasts from Cdc42-null mice migrate slightly slower than wild type cells but reintroduction of dominant-negative Cdc42 strongly inhibits this suggesting that the mutant also inhibits other pathways controlling cell polarity and migration (Czuchra et al., 2005).

Rho GTPases may facilitate the localisation of PTEN in some cells as neutrophils isolated from α -PIX-deficient mice or wild type neutrophils treated with the ROCK inhibitor Y-27632 are unable to polarise PTEN localisation (Li et al., 2005). However, PTEN

localisation in mammalian cell migration is controversial as it is not observed in all cells. Rac1 is believed to play a role in cell polarity through interaction with the Par complex. One function of this complex is in the regulation of tight junction assembly and apical-basal polarity through the activation of Rac1/Cdc42, but it is also believed to play a role in cell polarity in migration (reviewed in (Macara, 2004)). Rac1 could also establish cell polarity due to its intracellular localisation. Rac1 is localised to membrane ruffles and in a gradient towards the leading edge of growth factor-stimulated cells (Kraynov et al., 2000). Polarised Rac1 activity could lead to localised membrane protrusion and actin polymerisation through the mechanisms described below.

iii) Actin polymerisation and membrane protrusion

Studies using dominant negative Rac1 or Rac1-null mice have shown that Rac1 regulates membrane ruffling and lamellipodium formation in a variety of cell systems (Giehl et al., 2008; Nobes and Hall, 1999; Wells et al., 2004; Wheeler et al., 2006). It is thought that this can be facilitated through binding of the adapter protein IRSp53 to Rac1 to mediate interaction between Rac1 and WAVE2 (Abou-Kheir et al., 2008; Miki et al., 2000). Rac1 activates WAVE2 which binds Arp2/3 and leads to increased actin filament branching (Suetsugu et al., 1999; Suetsugu et al., 2001). WAVE2 is part of a complex with Abi1, Nap1 and Sra-1 (Innocenti et al., 2004; Kunda et al., 2003; Steffen et al., 2004). Formins are required for correct lamellipodium formation in some systems and it has previously been shown that formin-related protein (FRL) associates with Rac1 and is thought to participate in Rac1-induced actin polymerisation (Yang et al., 2007; Yayoshi-Yamamoto et al., 2000). PAKs also play a role in Rac1-induced lamellipodia formation by phosphorylating LIMK1/2 leading to cofilin inhibition and actin polymerisation which will be discussed in more detail later (Edwards et al., 1999).

Although RhoA is well characterized for its roles in stress fibre formation and tail retraction, it may also play a role in membrane ruffles and lamellipodia in some situations. Dominant negative (DN) RhoA but not DN Rac1 was shown to inhibit $\alpha 6 \beta 4$ -integrin-dependent membrane ruffling and lamella formation in adenocarcinoma cells (O'Connor et al., 2000). In agreement with this, RhoA localised to membrane ruffles in spreading endothelial cells (Menager et al., 1999) and active RhoA localised to protrusions as well as the rear of HeLa cells (Kurokawa and Matsuda, 2005). The presence of active RhoA in the

same location as active Rac1 and Cdc42 could be explained by overlapping GEFs, for example Vav2 activates RhoA, Rac1 and Cdc42 (Abe et al., 2000). The role of RhoA at the leading edge is unclear as it has also been suggested that RhoA restricts the formation of multiple lamellipodia (Worthylake and Burridge, 2003) and recent work in MTLn3 cells showed that ROCK inhibition leads to increased Rac1 activity and spreading in response to EGF which is rescued by Rac1 knockdown. This suggests that ROCK activity downstream of RhoA could negatively regulate Rac1 (El-Sibai et al., 2008). However it is possible that RhoA could increase lamellipodium formation through LIMK. ROCK has been shown to phosphorylate LIMK which would lead to cofilin inhibition and increased actin polymerisation (Sumi et al., 2001). Another possibility is that RhoA induces actin polymerisation through activation of the formin mDia. RhoA is believed to relieve autoinhibition of mDia1 (Lammers et al., 2005) while mDia and not ROCK was found to be stably associated with membrane ruffles (Kurokawa and Matsuda, 2005).

An expression screen of active Rho GTPases showed that RhoG, Rac2, Rac3, and Cdc42 can all induce lamellipodium formation whereas Wrch-1 (RhoU) induced formation of filopodia (Aspenstrom et al., 2004). RhoG probably induces lamellipodium formation through the activation of Rac1 (Katoh et al., 2006; Katoh and Negishi, 2003) and Cdc42 was originally characterized as inducing the formation of filopodia (Nobes and Hall, 1995) so it is likely that the effect of each Rho GTPase depends on the cell system, level of expression and the balance of other signalling pathways regulating actin dynamics. For example, as Rac1 and Cdc42 both interact with IRSp53, Cdc42 overexpression could lead to lamellipodia formation via crosstalk to the Rac1 pathway. Cdc42-mediated filopodium formation is believed to occur through its activation of WASP upstream of Arp2/3 (Section 1.2.1 and reviewed in Carlier et al., 1999). IQGAP, an actin binding adapter protein, is probably also involved in Cdc42-mediated WASP activation. IQGAP stimulates WASP/Arp2/3 based actin assembly and it has been suggested that stabilisation of GTP-bound Cdc42 could be involved (Le Clainche et al., 2007) and reviewed in (Brandt and Grosse, 2007). IQGAP could also be involved in actin polymerisation induced by Dial1 although it does not activate the actin-nucleation ability of Dial1 (Brandt et al., 2007).

iv) Adhesion turnover

Rho GTPases have been implicated in the control of new adhesions. Rac1 was shown to recruit $\alpha V\beta 3$ integrin to lamellipodia suggesting it plays a role in adhesion formation (Kiosses et al., 2001). A Rac1 effector implicated in focal adhesion dynamics is PAK1 and this will be discussed in more detail later.

v) Contractility

RhoA is believed to play a controlling role in myosin contractility. Inhibition of RhoA in macrophages led to an elongated morphology consistent with defective tail-retraction and active RhoA leads to the formation of stress-fibres (Allen et al., 1997; Ridley and Hall, 1992). RhoA is thought to exert control on contractility through its effector ROCK which can phosphorylate R-MLC at Ser19 (Amano et al., 1996). ROCK also phosphorylates the myosin-binding subunit of myosin phosphatase inhibiting it (Kawano et al., 1999). In this way RhoA/ROCK serves a dual role in the increase of myosin II incorporation into actin bundles and contractility. Recently a new member of the myosin phosphatase complex has been identified. Myosin phosphatase-Rho interacting protein, which is thought to recruit RhoA and is required for ROCK phosphorylation of myosin phosphatase (Riddick et al., 2008). Cdc42 can also regulate contractility through myotonic dystrophy kinase-related Cdc42-binding kinase (MRCK) in cooperation with ROCK to phosphorylate R-MLC (Wilkinson et al., 2005).

1.7: p21-activated kinases

p21-activated kinases (PAKs) are a family of serine/threonine kinases with an N-terminal regulatory region and a C-terminal kinase domain. Yeast PAK homologues (Ste20) were first characterized in 1992 where they were found to play a role in pheromone signalling (Leberer et al., 1992). Orthologues were subsequently identified in slime moulds, worms, flies and mammals (Figure 1.7) (reviewed in (Hofmann et al., 2004))

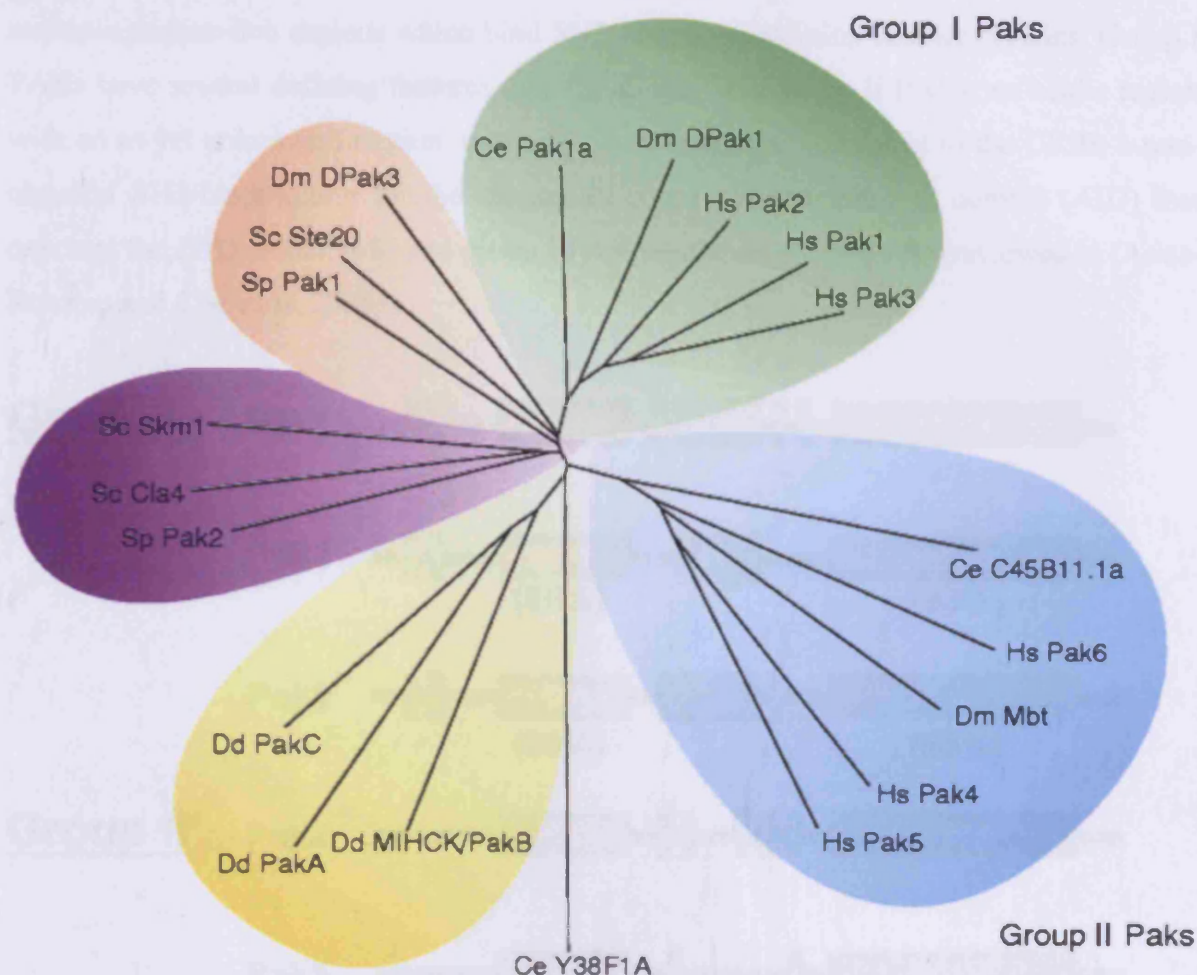


Figure 1.7: PAK orthologues. Phylogenetic tree of PAK orthologues showing isoforms from *Caenorhabditis elegans* (Ce); *Drosophila melanogaster* (Dm); *Dictyostelium discoideum* (Dd); *Saccharomyces cerevisiae* (Sc); *Homo sapiens* (Hs) and *Schizosaccharomyces pombe* (Sp). Colours represent groups of PAKs; Slime mould PAKs (yellow); yeast PAKs containing a PH domain (purple) and without a PH domain (orange) and two groups of PAKs from higher eukaryotes; group I (green) and group II (blue). Modified from (Hofmann et al., 2004).

Six PAKs have been identified in mammals, which are further classified into two groups with PAK1 - PAK3 in group I and PAK4 - PAK6 in group II. The group I PAKs share around 88% homology in the regulatory region and around 93% homology in the kinase domain. For group II PAKs the homology is around 60% in the regulatory region and 75% in the kinase domain (Figure 1.8).

All mammalian PAKs have a p21-binding domain (PBD) in the N-terminal region which contains a Cdc42/Rac interactive binding (CRIB) motif. The regulatory region also has

multiple proline-rich regions which bind SH3 domain-containing adapter proteins. Group I PAKs have several defining features that are absent from group II PAKs; an acidic region with an as yet unknown function; two proline-rich regions N-terminal to the CRIB; a non-classical SH3-binding site for the adapter PIX and an autoinhibitory domain (AID) that overlaps the PBD, which is key to group I PAK regulation (Figure 1.8) (reviewed in (Arias-Romero and Chernoff, 2008))

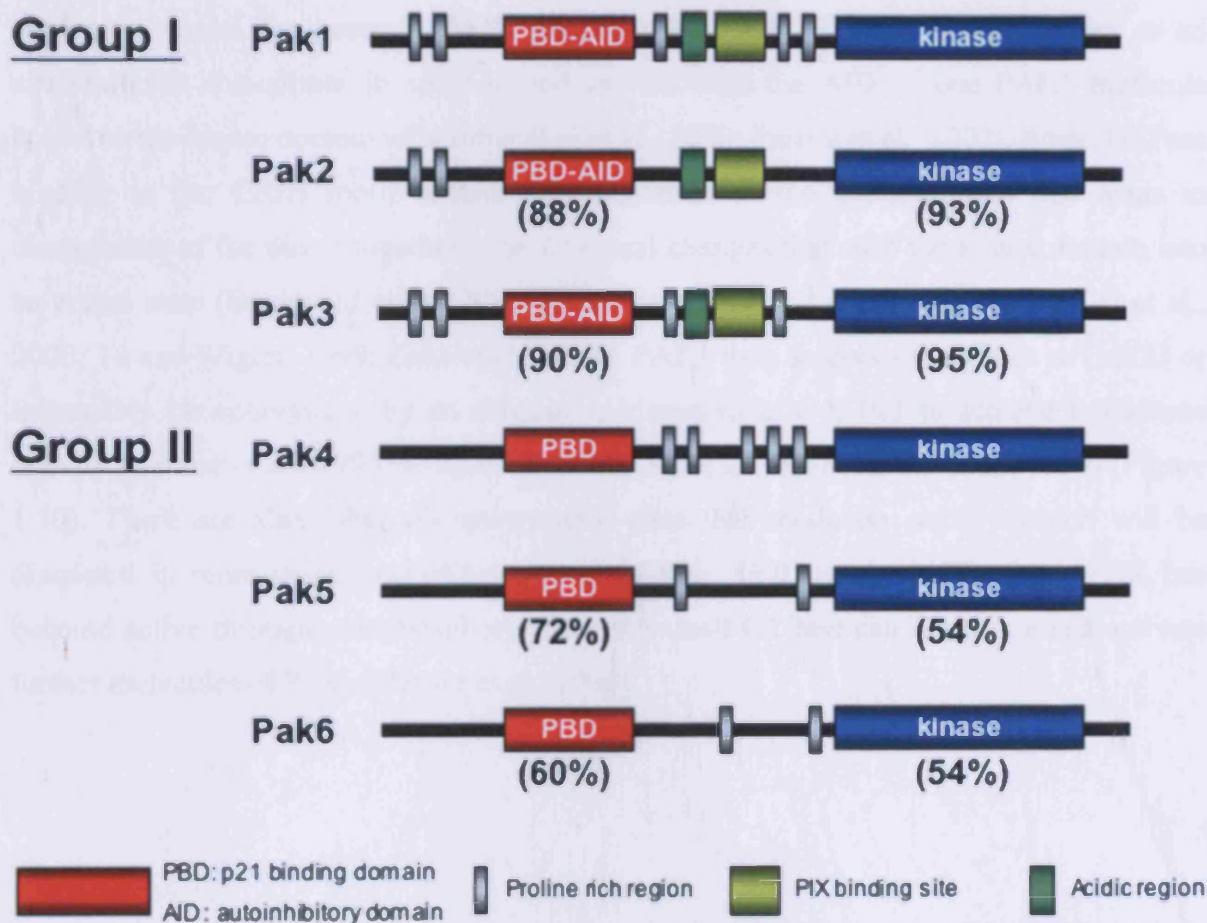


Figure 1.8: Schematic showing the conserved features of mammalian PAKs. Major features of group I and group II PAKs are shown including % homology between the N-terminal regulatory regions and between the kinase domains within each group compared to PAK1 for group I and PAK4 for group II. Taken from (Arias-Romero and Chernoff, 2008).

1.7.1: PAK regulation

1.7.1.1: Regulation of Group I PAKs

The presence of an autoinhibitory domain in group I PAKs means that their activation is distinct from that of the group II PAKs.

i) Regulation by small GTPases

Activation of PAK1 by Rac1 and Cdc42 has been extensively studied and is believed to provide a model for group I PAK activation by small GTPases. PAK1 exists as an autoinhibited homodimer in solution and *in vivo* with the AID of one PAK1 molecule bound to the kinase domain of another (Lei et al., 2000; Parrini et al., 2002). Small GTPase binding to the CRIB motif within the PBD releases the autoinhibition and leads to dissociation of the dimer together with structural changes that shift the kinase domain into an active state (Buchwald et al., 2001; Knaus et al., 1998; Leeuw et al., 1998; Lei et al., 2000; Tu and Wigler, 1999; Zhao et al., 1998). PAK1 then autophosphorylates at Thr423 or is possibly phosphorylated by an exogenous kinase such as PDK1 to achieve full kinase activity (Benner et al., 1995; King et al., 2000; Yu et al., 1998; Zenke et al., 1999) (Figure 1.10). There are also other phosphorylation sites that modulate activity which will be discussed in more detail later (Chong et al., 2001a; Gatti et al., 1999). Once PAK has become active through autophosphorylation, the small GTPase can dissociate and activate further molecules of PAK (Manser et al., 1994).

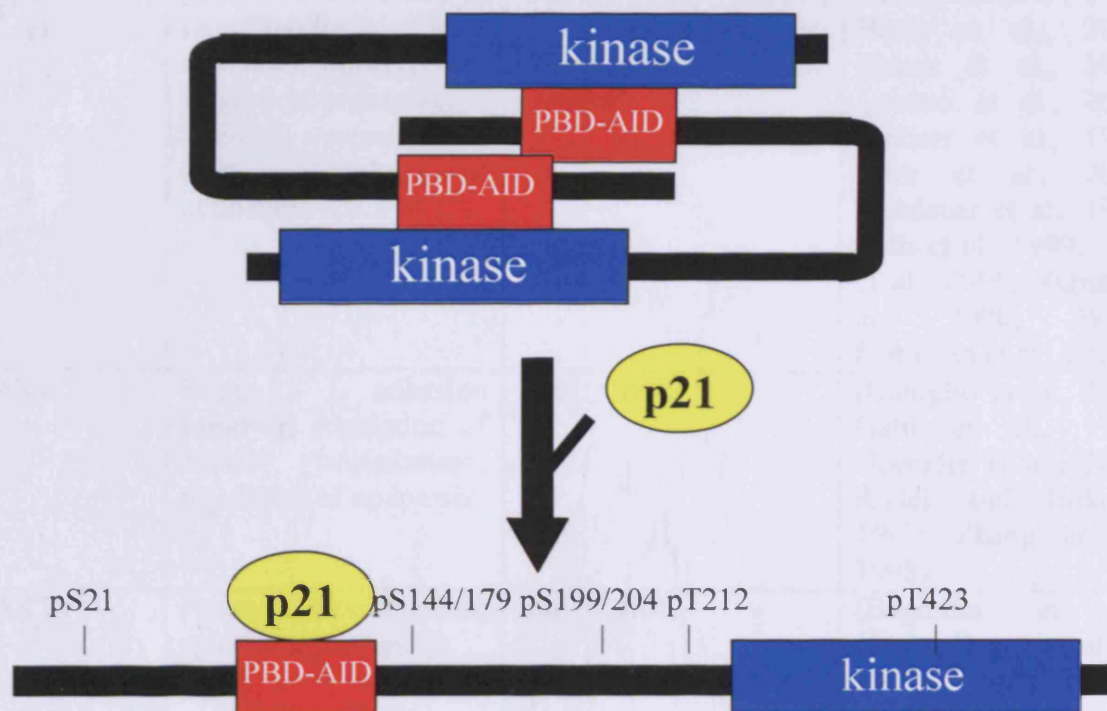


Figure 1.9: Mechanism of PAK activation. As described in the text, binding of a GTP-bound small GTPase to the PBD of a group I PAK relieves autoinhibition leading to autophosphorylation at T423 and full kinase activity. Subsequent phosphorylation at S21 by Akt blocks binding of the adapter Nck; autophosphorylation at S144/179 increases activity and at S199/204 blocks PIX binding and phosphorylation of T212 by ERK2 facilitates downstream ERK signalling.

PAK activation by Rac1 and Cdc42 have been well studied but other Rho GTPases have been shown to bind and activate group I and group II PAK isoforms (Table 1.2).

PAK isoform	Main suggested roles	Binding Rho GTPases	References
PAK1	Breakdown of adherens junctions; formation of lamellipodia; focal adhesion turnover; cell migration; regulation of actin organisation; spine morphogenesis; actin contractility.	Rac1; Rac2; Rac3; Cdc42; TCL (RhoJ); TC10 (RhoQ); Chp (RhoV); Wrch1 (RhoU)	(Abo et al., 1998; Aronheim et al., 1998; Boda et al., 2008; Knaus et al., 1998; Lozano et al., 2008; Manser et al., 1994; Mira et al., 2000; Neudauer et al., 1998; Sells et al., 1999; Tao et al., 2001; Vignall et al., 2000; Weisz Hubsman et al., 2007)
PAK2	Focal adhesion turnover; regulation of actin organization; regulation of apoptosis.	Rac1; Cdc42	(Coniglio et al., 2008; Gatti et al., 1999; Goeckler et al., 2000; Rudel and Bokoch, 1997; Zhang et al., 1998)
PAK3	Neuronal development; spine morphogenesis.	Rac1; Cdc42	(Bagrodia et al., 1995a; Bagrodia et al., 1995b; Boda et al., 2008)
PAK4	Cell migration; anti-apoptotic effects; regulation of actin organisation.	Rac1; Cdc42	(Abo et al., 1998; Ahmed et al., 2008; Gnesutta et al., 2001; Lu et al. 2003)
PAK5	Neurite outgrowth; Raf-1 signalling at mitochondria.	Cdc42; RhoD; RhoH	(Ching et al., 2003; Dan et al., 2002; Wu and Frost, 2006; Wu et al., 2008)
PAK6	Inhibition of androgen receptor signaling.	Currently unknown	Schrantz et al., 2004;

Table 1.2: Rho GTPases shown to bind mammalian PAK isoforms. Rho GTPases also shown to activate kinase activity of the relevant PAK are shown in green.

ii) GTPase-independent regulation

PAK activation by GTPase-independent mechanisms has been observed. PAK2 is cleaved by caspase 3 yielding an active kinase domain fragment which is thought to regulate morphological changes during apoptosis in Jurkat cells (Rudel and Bokoch, 1997). PAK1

has been shown to be activated by filamin-A binding to the CRIB domain in a similar way to GTPase binding (Vadlamudi et al., 2002).

SH3-containing adaptor proteins can further regulate the intracellular localisation of PAK. Both Grb2 and Nck recruit PAK to activated receptor tyrosine kinases such as the epidermal growth factor receptor (Galisteo et al., 1996; Puto et al., 2003). Binding of α -PIX or β -PIX to PAK in complex with GIT recruits it to focal adhesions and this will be discussed in more detail later (ten Klooster et al., 2006).

iii) Regulation by phosphorylation.

Group I PAKs are further regulated by phosphorylation at multiple sites (Figure 1.9). Phosphorylation of PAK1 at T423 in the kinase domain by autophosphorylation or PDK1 in the presence of sphingosine is required for full PAK activation (King et al., 2000; Zenke et al., 1999). PAK2 and PAK3 contain an homologous site (T402 and T421 respectively), phosphorylation of which is also required for full kinase activity (Chong et al., 2001a; Walter et al., 1998; Zenke et al., 1999).

Autophosphorylation of another conserved site, S144/S149 in PAK1 or S139 in PAK3, also affects kinase activity (Chong et al., 2001a). PAK2 contains this site at S141 but it may be constitutively phosphorylated (Zhan et al., 2003)

Other phosphorylation sites affect binding of adapters and the persistence of PAK activation. For example, Akt phosphorylation of PAK at serine 21 by Akt blocks Nck binding (Zhou et al., 2003) and phosphorylation at serine 199 and 204, both autophosphorylation sites, blocks PIX α binding (Mott et al., 2005). Phosphorylation at S199/204 has previously been used as an experimental indicator of PAK1 activation but phosphorylation by another kinase cannot be ruled out. PAK1 is also phosphorylated by ERK2 at threonine 212 which is believed to facilitate downstream mitogen activated protein kinase (MAPK) signalling (Sundberg-Smith et al., 2005).

1.7.1.2: Regulation of Group II PAKs

The mechanisms of group II PAK activation are not well known and few Rho GTPase binding partners have been identified (Table 1.2). It was shown that Cdc42 binding to

PAK4 does not increase kinase activity and it was suggested that group II PAKs do not contain an AID (Abo et al., 1998). However, it has now been proposed that PAK5 is activated by Cdc42 and may possess an N-terminal AID but this finding remains controversial (Ching et al., 2003). PAK4 and PAK6 lacking the N-terminal region are more active than the full length isoforms suggesting that the N-terminal region negatively regulates their kinase activity (Abo et al., 1998; Yang et al., 2001). Group II PAKs contain an autophosphorylation site homologous to the PAK1 T423 site but it is a serine rather than a threonine. In PAK4, mutation of this site (S474) to methionine blocks kinase activity (Abo et al., 1998) and mutation of S474 to glutamine increases PAK4 activity towards a peptide encompassing the S474 region suggesting that phosphorylation might increase activity although it is not known if this occurs *in vivo* (Callow et al., 2002).

Although the mechanisms of activation are unclear, the pathways leading to Group II PAK activation are beginning to be revealed. PAK4 binds Cdc42, and is activated following HGF stimulation via PI3K in a Cdc42-independent manner in MDCK cells (Wells et al., 2002). PAK4 also binds keratinocyte growth factor (KGF) receptor and is thought to mediate anti-apoptotic effects of KGF (Lu et al., 2003). Like the group I PAKs, group II PAKs are also regulated by localization as they contain adapter binding sites. Activated PAK4 is targeted to the cell periphery (Wells et al., 2002) and PAK5 is localized to mitochondria in a manner blocked by truncation of the CRIB domain. It was also suggested that the binding of Cdc42 targets PAK5 to a different subcellular localization than RhoD binding (Wu and Frost, 2006). PAK6 binds to androgen receptor (AR) and translocates to the nucleus with AR in androgen-stimulated cells (Yang et al., 2001). PAK6 has also been shown to be activated by MAP kinase kinase 6 and p38 MAP kinase (Kaur et al., 2005). Through these different pathways, it seems likely that group II PAKs have distinct functions from each other.

1.7.2: PAK expression

PAK isoforms are differentially expressed in normal tissues and can be upregulated in some cancers. PAK1 was found to be amplified, overexpressed or stabilised in breast, ovarian, colon, bladder and brain cancers as well as neurofibromatosis and T-cell lymphoma. PAK4 has been found amplified and overexpressed in pancreatic cancer and many cancer cell lines (Callow et al., 2001 and reviewed in Kumar et al., 2006).

Knockout mice have been generated for five of the PAKs: PAK1 knockout mice have immune defects, PAK2 and PAK4 knockouts are embryonic lethal, PAK3 knockout mice have neuronal defects and PAK5 knockout mice have no detectable phenotype (Table 1.3). For this reason it was suggested that PAK5 is redundant but recent evidence shows that PAK5 probably does have a role in cells: It is the only PAK shown to bind to RhoD and RhoH (Wu and Frost, 2006). In the case of PAK3, loss of function in humans is found in X-linked mental retardation syndrome (Peippo et al., 2007).

PAK isoform	Mouse knockout phenotype	References
PAK1	Immune defects	(ten Klooster et al., 2006)
PAK2	Embryonic lethal and defective MAPK signalling	(Arias-Romero and Chernoff, 2008)
PAK3	Learning and memory deficiency, and abnormal synaptic plasticity	(Meng et al., 2005)
PAK4	Embryonic lethal due to heart and nervous system defects	(Qu et al., 2003)
PAK5	No detectable phenotype	(Li and Minden, 2003)

Table 1.3: Phenotypes of PAK knockout mice.

1.7.3: Roles of PAK in cell migration

PAKs mediate a link between many Rho GTPases and cytoskeletal rearrangement, and take part in each of the stages of cell migration.

i) Breakdown of cell junctions

It was described that N-cadherin-regulated adhesion inhibits Rac1 and Cdc42 suggesting that it would also locally inhibit PAK activation (Charrasse et al., 2002). But in a different study, E-cadherin ligation was shown to recruit and stimulate Rac1 implying that Rac1-mediated signalling could be involved in junctional stability (Kovacs et al., 2002). Until recently the role of PAK1 in cell junctional breakdown had not been described, but it is now believed that PAK1 can mediate the breakdown of adherens junctions downstream of Rac1 (Lozano et al., 2008). Another possible role for PAK1 in junctional breakdown is

through the transcriptional repression of E-cadherin and occludin via phosphorylation of Snail, but this could also alter the expression of many other proteins making it difficult to draw specific conclusions (Yang et al., 2005). It is unknown whether PAKs influence the breakdown or formation of tight junctions. A possible link could be by PAK1 or PAK4-mediated phosphorylation of GEF-H1, a Rho GEF that regulates junctional permeability, but this link has not been investigated (Benaï-Pont et al., 2003; Callow et al., 2005; Zenke et al., 2004).

ii) Cell polarity and focal adhesion turnover

PAK1 is recruited to the leading edge of motile neutrophils and fibroblasts, and dominant negative PAK1 blocked chemotaxis towards the chemokine CXCL1 in RBL-2H3 mast cells suggesting that it plays a part in polarised cell signalling and persistence of the leading edge (Dharmawardhane et al., 1999; Dharmawardhane et al., 1997; Wang et al., 2002a). As previously mentioned, it was found that activation of Cdc42 in an α -PIX/PAK1-dependent manner is required for directional migration of leukocytes including actin polymerisation and the exclusion of PTEN from the leading edge (Li et al., 2003). PAK1 or PAK2 are thought to form a complex with G protein-coupled receptor kinase-interacting protein (GIT1/2) through mutual interaction with α or β -PIX. This complex localizes to focal adhesions by the binding of GIT to FAK or paxillin (Bagrodia et al., 1999; Manser et al., 1998; Nayal et al., 2006; Zhang et al., 2008; Zhao et al., 2000) (Figure 1.11). In this way, GIT/PIX/PAK would be recruited to the sites of nascent adhesion formation in the leading edge or ruffles of motile cells.

Localization of the complex can be influenced by each of PAK, GIT or PIX (Manabe et al., 2002; Stofega et al., 2004; Webb et al., 2006; Za et al., 2006). Complex binding is believed to be further regulated by phosphorylation. It was described that phosphorylation of paxillin at Ser273 increases binding of GIT1 but recent *in vitro* data disputes this so it remains to be confirmed (Nayal et al., 2006; Zhang et al., 2008). GIT1 phosphorylation was also investigated. GIT1 contains two serine residues in the paxillin binding site at amino acid 709 and 710. It was suggested that phosphorylation of Ser709 in GIT1 would increase binding to paxillin, as mutation of Ser709 to alanine impaired protrusion and paxillin binding (Webb et al., 2006). However, mutation to glutamic acid to mimic phosphorylation had little effect. Furthermore, in the same study it was suggested that Ser709 in GIT1 might

be a PAK1 substrate. A peptide surrounding Ser709 was phosphorylated by active but not kinase dead PAK1 and S709A substitution decreased phosphorylation by active PAK1. However, the S709A peptide was still phosphorylated around 10-fold more by active PAK1 than kinase dead PAK1. Taken together, it seems more likely that Ser710 is a PAK1 substrate and regulates binding of GIT1 to paxillin.

GIT/PIX/PAK coupling to focal adhesions is thought to stimulate focal adhesion turnover together with kinase signalling to phosphorylate ERK (Stockton et al., 2007; Yin et al., 2005; Zhao et al., 2000). However, there is some conflicting evidence as to whether both GIT1 and GIT2 are involved. GIT2 knockdown led to smaller focal adhesions and increased spreading in HeLa cells (Frank et al., 2006). It seems that GIT2 may be differentially regulated to GIT1 as knockout of some focal adhesion proteins including FAK and paxillin blocked focal adhesion localization of GIT2 but not GIT1 in MEFs (Brown et al., 2005). The roles for PAK in focal adhesion turnover are still unclear as PAK has been implicated in both formation and disassembly of focal adhesions (Kiosses et al., 1999). Evidence also suggests that PAK1 could be required for FAK phosphorylation in certain situations and it was recently suggested that knockdown of PAK2 leads to an increase in the size of focal adhesions, hence decreased turnover (Coniglio et al., 2008; Jung et al., 2004). PAK3 might directly bind and phosphorylate paxillin although this is controversial and has not been subsequently investigated (Hashimoto et al., 2001).

The ability of the GIT/PIX/PAK complex to regulate focal adhesion turnover and protrusion is likely to be mediated through Rac1/Cdc42 and PAK kinase activity. α and β -PIX are Rac1/Cdc42 GEFs and β -PIX has been shown to be important for the localization of Rac1 to membrane ruffles and associated Rac1 activity. This was blocked by PAK, presumably by competing for the Rac1 binding site in β -PIX (ten Klooster et al., 2006). A model was proposed where GIT/PIX/PAK localization to focal adhesions and subsequent PAK activation would lead to PAK phosphorylation at S199/204 and dissociation from β -PIX. This would allow β -PIX to bind Rac1 and mediate its activation (Figure 1.11). Rac1-mediated activation of PAK would serve as a positive feedback loop in this model leading to further PAK autophosphorylation. Phosphorylation of GIT1 and paxillin by PAK could also be a source of positive feedback by increasing the recruitment of GIT1 to paxillin. Active Rac1 could lead to membrane protrusion via WAVE (Chapter 1,

Section 1.6.2) or via PAK as described in section iii below. PAK1 was also shown to be activated by GIT1 independently of Cdc42 or Rac1 binding to PAK1 but this could occur through another Rho GTPase (Loo et al., 2004).

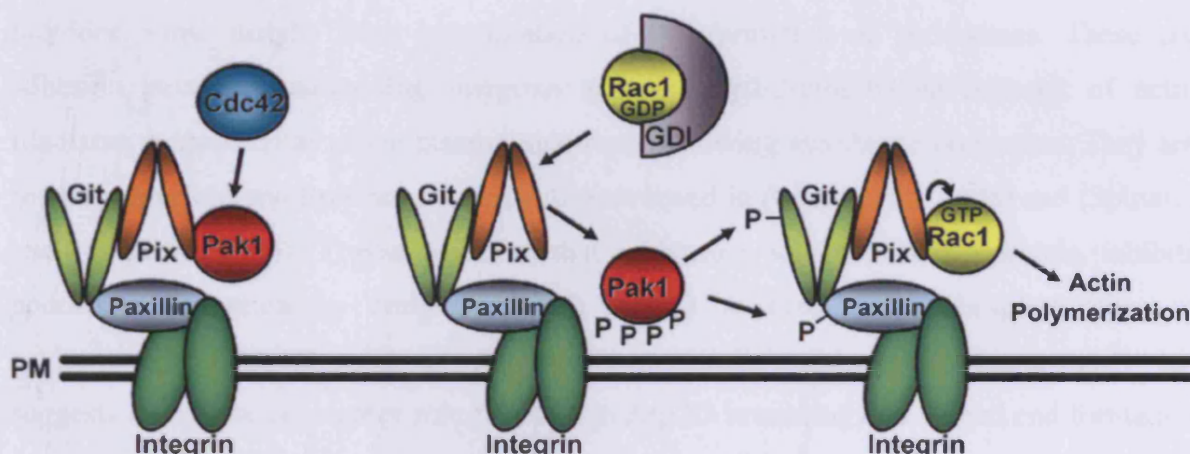


Figure 1.10: Model for the control of Rac1, Cdc42 and PAK1 activation by GIT/PIX. GIT/ β -PIX/PAK1 targeting to paxillin and localised Cdc42 activity at the leading edge of cells leads to PAK1 activation and phosphorylation at S199/204 releasing it from β -PIX as well as phosphorylation at other sites. Release of PAK1 frees β -PIX to bind and activate Rac1 and downstream signalling leads to actin polymerisation. Phosphorylation of GIT1 and paxillin by PAK1 would increase the recruitment of GIT1/ β -PIX in a positive feedback mechanism. Adapted from (ten Klooster et al., 2006).

iii) Actin polymerisation

PAK-dependent induction of actin polymerisation is thought to be mediated through LIM-kinase 1 (LIMK1). PAK1, PAK2 and PAK4 have all been shown to activate LIMK1 (Dan et al., 2001; Edwards et al., 1999; Misra et al., 2005). LIMK1 phosphorylates and inactivates actin filament severing and depolymerisation by cofilin leading to an increase in actin filaments (Arber et al., 1998). PAK1, PAK2 and PAK4 have also been described to phosphorylate LIMK2 although data has not yet been published (Bokoch, 2003). Another protein implicated in PAK-dependent actin dynamics is Filamin-A, an actin cross-linking protein which links actin filaments in a flexible manner with no defined angle (reviewed in (Popowicz et al., 2006)). It has been described that filamin-A is required for ruffle formation by overexpressed PAK1 and it was shown that filamin-A binds to PAK1 at the CRIB domain and stimulates PAK1 activity, probably in a similar way to Rho GTPase binding. In turn, PAK1 phosphorylates Filamin-A at Ser2152 (Vadlamudi et al., 2002). As

yet no more is known of the significance of this study and it would be interesting to know the role endogenous filamin-A plays in PAK-mediated actin polymerisation.

Whether PAK regulates the Arp2/3 complex is poorly understood but recent work has provided some insight from investigation of the formation of podosomes. These are adhesion structures containing integrins and an Arp2/3-crosslinked network of actin filaments perpendicular to the plasma membrane allowing membrane protrusion. They are found in myeloid and Src-transformed cells (reviewed in (Block et al., 2008) and (Spinardi and Marchisio, 2006)). It was described that caldesmon, an actin binding protein, inhibits podosome formation by competing with Arp2/3 to bind actin. Phosphorylation of caldesmon by PAK1/2 enhances this inhibition (Morita et al., 2007). Interestingly this suggests a negative regulatory role for PAK in Arp2/3 branching and barbed end formation which is surprising considering the role of Arp2/3 in lamellipodium formation. Nonetheless, studies using alanine and glutamic acid mutations of the PAK phosphorylation site in caldesmon suggest that phosphorylation of caldesmon by PAK could play a role in the actin dynamics of lamellipodia and stress fibres although both mutations impaired cell migration (Eppinga et al., 2006). Further work is clearly required to determine the role of caldesmon phosphorylation by PAK in cell migration.

iv) Cell contractility

PAK1 and PAK2 have been shown to be able to phosphorylate R-MLC at Ser19 which leads to increased contraction (Chew et al., 1998; Ramos et al., 1997; Rudrabhatla et al., 2003; Zeng et al., 2000). Conversely, PAK1 and PAK2 can also inhibit myosin contraction by phosphorylating and inhibiting MLCK which normally phosphorylates R-MLC at both Thr18 and Ser19 (Goeckeler et al., 2000; Ikebe and Hartshorne, 1985; Ikebe et al., 1986; Sanders et al., 1999). The relevance of the dual role of PAK1/2 is unclear but it seems likely that the functional outcome in terms of contractility would depend on the cellular localization and relative local concentrations of PAK1/2, myosin and MLCK. As previously described, in some cell types there appears to be a gradient of myosin II incorporation onto actin bundles in migrating cells from a low concentration of myosin at the leading edge to a high concentration in the tail (Svitkina et al., 1997; Verkhovsky et al., 1995, 1997). The dual role of PAK1/2 could facilitate this. Active PAK1/2 localised to the leading edge would phosphorylate R-MLC at Ser19 initiating the incorporation of myosin

into actin bundles. At the same time, MLCK would be inhibited by PAK blocking diphosphorylation of R-MLC. Retrograde flow moving the actin network towards the rear of the cell would carry phospho-Ser19 myosin with it and MLCK would become diphosphorylated and active. Expression studies in rat embryo fibroblasts (REF-52), bovine pulmonary artery endothelial cells and HeLa cells showed that the 130 kDa isoform of MLCK is localized throughout the cell with localized regions of higher concentration in ruffles or in the perinuclear region of REF-52 and HeLa cells (Blue et al., 2002; Poperechnaya et al., 2000). In a migrating cell PAK1/2 activity in the leading edge could lead to a gradient of active MLCK towards the rear of the cell which would phosphorylate R-MLC at both Thr18 and Ser19 further incorporating myosin into actin bundles and stimulating contraction to drive the cell forwards. The distribution of phospho-S19 R-MLC and diphosphorylated R-MLC in motile fibroblasts and COS7 cells seem to support this model as S19 R-MLC was enriched at the leading edge and diphosphorylated R-MLC localised at stress fibres (Komatsu and Ikebe, 2004; Matsumura et al., 1998). However there was some enrichment of S19 R-MLC in the tail of fibroblasts, perhaps due to dephosphorylation as stress fibres disassemble.

PAK1 and PAK4 have been described to play a role in contractility by phosphorylating the RhoGEF GEF-H1 at Ser885 and it was suggested that this might inhibit its RhoA GEF activity and increase activity on Rac1 although further evidence is required (Callow et al., 2005; Zenke et al., 2004). It has been shown that GEF-H1 is active towards RhoA, RhoB, RhoC and Rac1 but not Cdc42 *in vitro* but it is not known whether this specificity is the same *in vivo* (Ren et al., 1998). Inhibition of GEF-H1 could lead to a decrease in GTP bound RhoA which would decrease contractility through RhoA/ROCK.

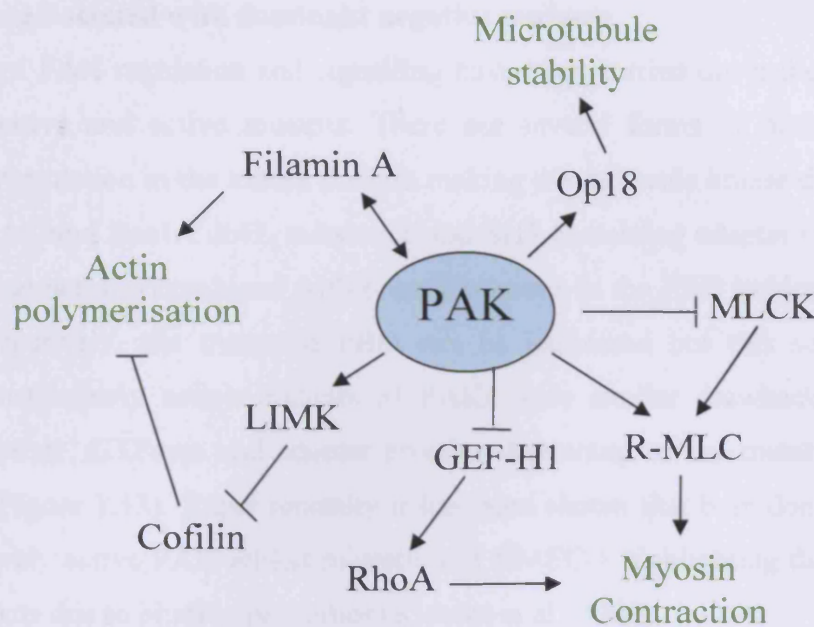


Figure 1.11: Control of the cytoskeleton by PAKs. As described in the text PAK isoforms can increase microtubule stability through inhibition of Op18; increase actin polymerisation through LIMK and Filamin-A dependent pathways; and either suppress or stimulate myosin contractility through R-MLC or RhoA.

1.7.4: Roles of PAKs in MAPK signalling

The original identification of Ste20 as a kinase in the yeast pheromone pathway suggested that PAKs could regulate MAPK signalling in mammalian cells (Leberer et al., 1992). A role for PAK1 in the Raf1/MEK/ERK pathway was described when PAK1 was found to phosphorylate Raf1 at Ser338, an activating phosphorylation, and MEK1 at Ser298 which is believed to prime MEK1 for phosphorylation by Raf1. (Beeser et al., 2005; Chong et al., 2001b; Coles and Shaw, 2002; Eblen et al., 2002; Slack-Davis et al., 2003). PAK4 has also been shown to activate the ERK pathway (Cammarano et al., 2005) and initial characterization of PAK5 showed that it can activate the c-Jun N-terminal kinase (JNK) pathway (Dan et al., 2002). Subsequently PAK1 and PAK2 were also implicated in the activation of the p38 MAPK pathway downstream of CXCL12 (Rousseau et al., 2006).

As well as transcriptional regulation, it has been suggested that p38 α could be involved in cell motility as pharmacological inhibition of p38 MAP kinases impaired β -PIX-induced membrane ruffling (Lee et al., 2001), and p38 MAP kinase-deficient mouse embryonic fibroblasts have impaired migration (Rousseau et al., 2006).

1.8: Problems associated with dominant negative mutants

Most studies of PAK regulation and signalling have been carried out using expression of dominant negative and active mutants. There are several forms of dominant negative PAK1; a point mutation in the kinase domain making the molecule kinase dead is used but it is still able to bind Rac1/Cdc42, substrates and SH3-containing adapter proteins (Figure 1.13). This is sometimes combined with point mutations in the PBD to block Rac1/Cdc42 binding. Alternatively, the truncated PBD can be expressed but this sequesters small GTPases. Constitutively active mutants of PAKs have similar drawbacks as they can sequester substrate, GTPases and adapter proteins depending on the mutations present to prevent this (Figure 1.13). Experimentally it has been shown that both dominant negative and constitutively active PAK inhibit migration of HMEC-1 highlighting the possibility of unwanted effects due to binding properties (Kiosses et al., 1999).

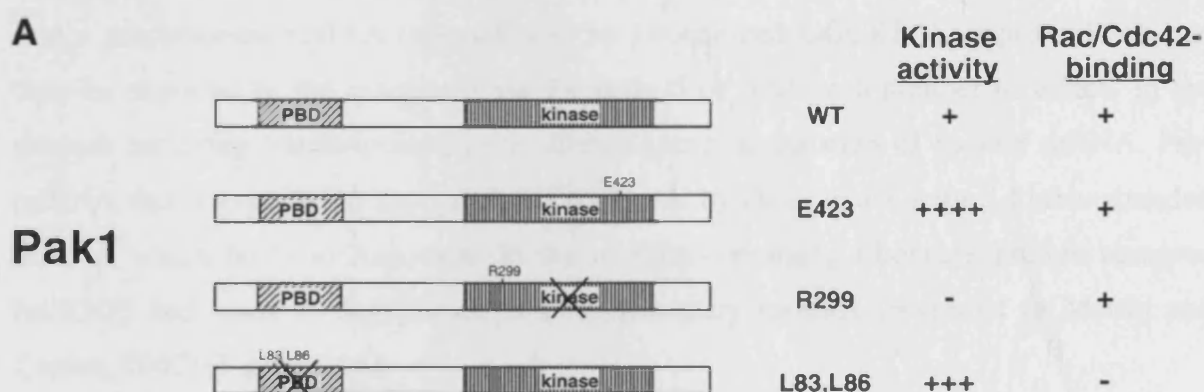


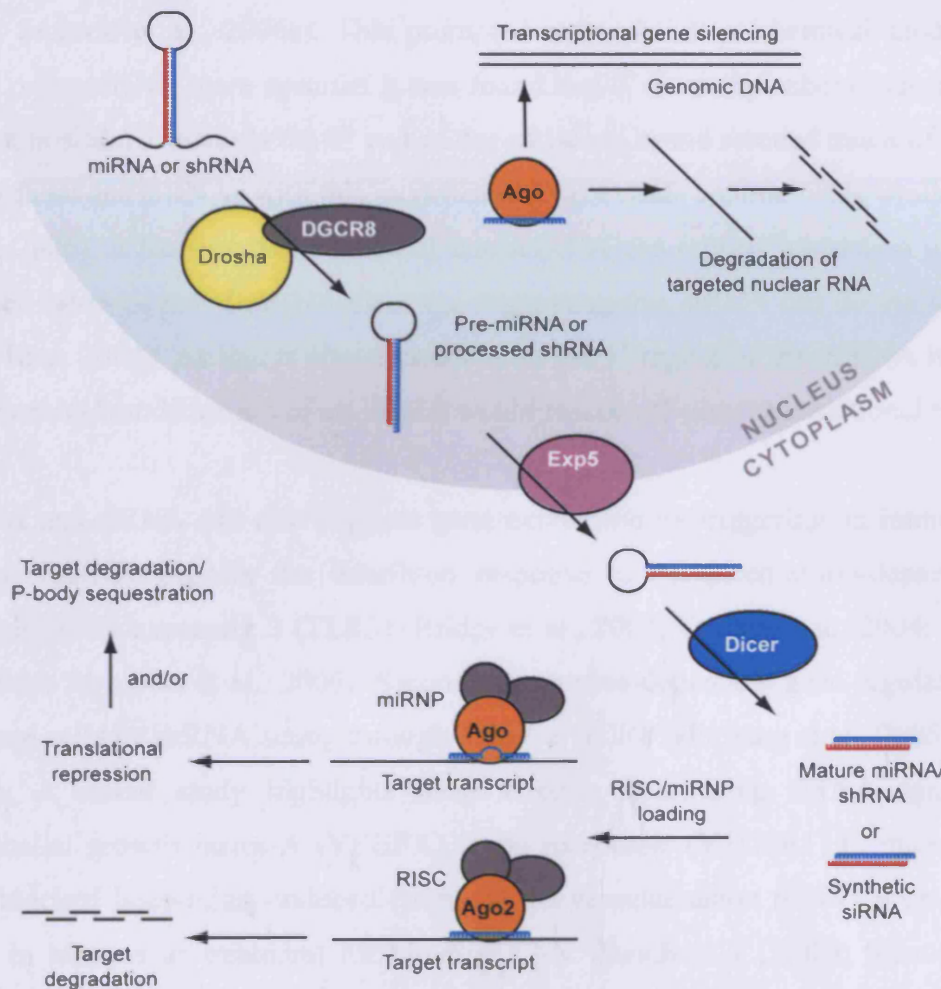
Figure 1.12: Schematic showing wild type, constitutively active and dominant negative forms of PAK1. Point mutations are shown together with relative activity of the construct and Rac1/Cdc42 binding status. (Taken from (Sells et al., 1999))

Dominant negative and constitutively active Rho GTPases have a similar problem because they sequester active Rho GEFs, GAPs and effectors thereby inhibiting signalling by other Rho GTPases which share the same regulatory proteins.

1.9: RNA interference (RNAi)

RNAi provides an alternative to the use of dominant negative mutants and also allows the study of proteins and signalling pathways where a dominant negative is not available. Its use has increased rapidly because it is simple to perform and can be used to screen families of genes for function in specific assays. RNAi makes use of a previously unknown gene regulation pathway in cells where the presence of double stranded RNA (dsRNA) in the cytoplasm leads to the down-regulation of mRNA containing an identical sequence. The effect of double stranded RNA was first discovered in plants (reviewed in (Vaucheret, 2006)) and it has subsequently been shown to have the same effect in a variety of species including *Caenorhabditis Elegans*, *Drosophila melanogaster*, *Mus musculus* and *Homo sapiens* (Caplen et al., 2001; Fire et al., 1998; Kennerdell and Carthew, 1998). It is thought that RNAi is both an endogenous mechanism for gene regulation and an intracellular defence against viruses that have a dsRNA genome. In gene regulation, an RNA is expressed that forms a short hairpin structure and is subsequently processed in the nucleus into a precursor-microRNA (pre-miRNA) by Drosha and DGCR8. This pre-miRNA can then be exported to the cytoplasm via Exportin 5 or mediate a number of effects in the nucleus including transcriptional gene silencing and degradation of nuclear mRNA. Pre-miRNA that is exported to the cytoplasm is cleaved by Dicer into a mature double-stranded miRNA which binds to Argonaute in the miRNA-containing ribonucleoprotein complex (miRNP) and leads to degradation of complementary mRNA (reviewed in Martin and Caplen, 2007) (Figure 1.14).

Experimentally, transient gene knockdown can be accomplished by transfecting a short dsRNA oligomer usually consisting of 19 matched nucleotides with additional 3' dinucleotide overhangs. Once in the cytoplasm, Argonaute 2 (Ago2) recognizes the 3' overhang and incorporates the siRNA into the RNA-induced silencing complex (RISC), which then degrades complementary mRNA (Figure 1.14). The methods used for the transfection of cells with siRNA are the same as those used to transfect DNA i.e. electroporation and lipid-based transfection. Stable knockdown or inducible knockdown can also be achieved using plasmid-based expression of a short-hairpin RNA (shRNA) which is processed in the same way as endogenous miRNA.



AR Martin SE, Caplen NJ. 2007.
Annu. Rev. Genomics Hum. Genet. 8:81–108

Figure 1.13: The miRNA/siRNA pathway: See text for details. (Taken from (Martin and Caplen, 2007))

1.10: Problems with RNAi

Although RNAi was originally believed to be highly specific, it became clear that the introduction of siRNA into cells causes degradation of multiple mRNAs in addition to the target (Jackson et al., 2003). Studies into the mechanism of miRNA showed that knockdown can be mediated through complementarity between the ‘seed region’ of the miRNA and the 3’ untranslated region of an mRNA (Lim et al., 2005; Lewis et al., 2003). It was subsequently shown that the seed region of an siRNA can mediate off-target knockdown in the same way in a concentration-dependent manner (Birmingham et al.,

2006; Jackson et al., 2006a). This prompted research into a chemical modification that could make siRNA more specific. It was found that 2'-*O*-methyl ribosyl substitution of the base at position 2 towards the 5' end of the antisense strand rescued much of the off-target knockdown and siRNAs with this modification were made commercially available (Jackson et al., 2006). It has also been reported that miRNA can repress translation of protein in a manner independent of mRNA cleavage suggesting that siRNA can do the same (Doench and Sharp, 2004). As this is also dependent on the 5' region of the miRNA it seems likely that chemical modification of an siRNA would rescue off-target translational repression.

shRNA and siRNA can also regulate gene expression by triggering an immune response. Firstly, dsRNA triggers the interferon response in a concentration-dependent manner through toll-like receptor 3 (TLR3) (Bridge et al., 2003; Kariko et al., 2004; Persengiev et al., 2004; Reynolds et al., 2006). Secondly, sequence-dependent gene regulation occurs in immune cells by dsRNA acting through TLR7 or TLR8 (Hornung et al., 2005; Judge et al., 2005). A recent study highlights these effects, trials using siRNA against vascular endothelial growth factor-A (VEGFA) or its receptor-1 (VEGFR1) in mice showed that they blocked laser-injury-induced choroidal neovascularisation (CNV) prompting clinical trials in humans as treatment for blinding CNV (Reich et al., 2003; Shen et al., 2006). However, it has now been shown that the effect is not sequence-dependent but occurs as a result of the effect of siRNA on TLR3 (Kleinman et al., 2008)

dsRNA can have further non-specific effects by inhibiting the endogenous miRNA pathway through the saturation of dsRNA binding proteins. It is thought that this is mainly a problem with overexpression of an shRNA as it appears to involve saturation of Exportin5, the protein responsible for the nuclear export of pre-miRNA which is not involved in the siRNA pathway (Grimm et al., 2006; Yi et al., 2005). However, saturating effects of high siRNA concentrations cannot be ruled out.

The concentration-dependent nature of many off-target effects highlights the need to use as low an effective concentration of siRNA as possible in experiments. It is also important to replicate an experimental result with at least two different siRNA sequences targeting the same mRNA to control for sequence-dependent off-target effects. A scrambled non-

targeting siRNA or shRNA should be used as a control for sequence-independent effects and chemical inhibition of the target should be used where available to corroborate results.

1.11: Hepatocyte growth factor signalling

Hepatocyte growth factor (HGF) is a protein in the plasminogen-related growth factor family. The HGF receptor c-Met is a receptor tyrosine kinase which is expressed as a 150 kDa precursor and then glycosylated and cleaved into a 50 kDa extracellular α chain linked by disulphide bonds to a 140 kDa β chain with a single transmembrane domain (reviewed in Ma et al., 2003 and Birchmeier et al., 2003) (Figure 1.15). Binding of HGF to c-Met leads to tyrosine kinase activation and trans-phosphorylation of Tyr1234 and Tyr1235 in the kinase domain. Further autophosphorylation at Tyr1349 and Tyr 1356 creates a docking site for adaptor proteins.

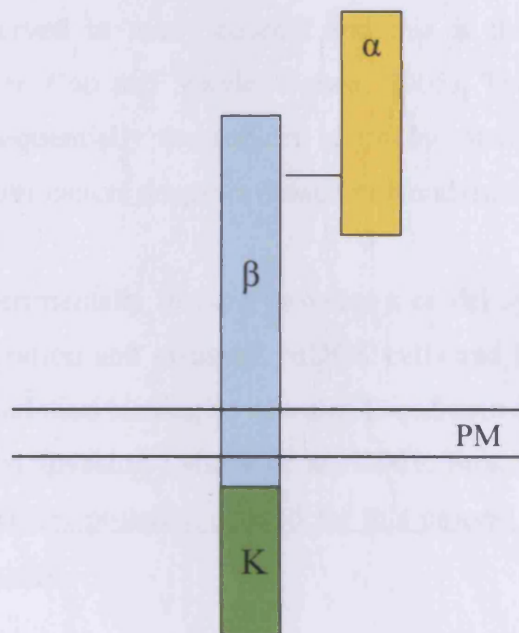


Figure 1.14: Schematic diagram of c-Met. As described, c-Met consists of an extracellular α -chain linked to a β -chain with an intracellular tyrosine kinase domain (K).

Signalling downstream of HGF is highly diverse, HGF binding to c-Met leads to the activation of PI-3-K and Ras, the downstream pathways of which cause a variety of cell responses including altered transcriptional regulation, cell scattering and chemotaxis, increased proliferation and invasion (Ahmed et al., 2008; Behrens et al., 1991; Ramos-Nino et al., 2008; Rosen et al., 1993; Tulasne et al., 2002). A well defined readout of HGF stimulation in c-Met-expressing cells is the phosphorylation and activation of MAP kinases

such as ERK1 and ERK2. Signalling to these pathways is not thought to be limited to c-MET at the plasma membrane. HGF stimulates the endocytosis and degradation of c-Met and c-Met signalling to ERK1 and ERK2 continues after endocytosis (Hammond et al., 2001; Kermorgant et al., 2003; Kermorgant et al., 2004). HGF-induced cell migration is likely to be mediated by Rho GTPases such as Cdc42 and Rac1 (Bosse et al., 2002), but as yet the contribution of each individual Rho GTPase to HGF-induced membrane ruffling and cell migration has not been defined. HGF has been shown to activate PAK1 and PAK4 in MDCK cells suggesting that PAKs could mediate cell migration downstream of HGF (Royal et al., 2000; Wells et al., 2002). Further investigation implicated a PAK4/LIMK1 pathway in cancer cell migration which could be of relevance to cancer metastasis (Ahmed et al., 2008).

Upregulated c-Met/HGF signalling caused by overexpression of c-Met or a mutant form of c-Met has been observed in many cancers and this is thought to correlate with poor prognosis (reviewed in Gao and Vande Woude, 2005). The roles HGF plays in cancer progression are consequentially the subject of intense study and c-Met is an important target for a range of anti-cancer drugs (reviewed in Knudsen and Vande Woude, 2008).

The use of HGF experimentally *in vitro* provides a model system for the investigation of cell signalling in migration and invasion. MDCK cells and DU145 cancer cells scatter in response to HGF stimulation leading to a loss of E-cadherin from junctions combined with increased motility and invasion (Miura et al., 2001; Stoker and Perryman, 1985). The identification of individual proteins required for this process could reveal potential targets for new anti-cancer drugs.

1.12 Aims

PAKs have been implicated in many signalling pathways associated with increased cell migration and they have been shown to be upregulated in many cancers. This implies that PAKs could be potential therapeutic targets for anti-metastatic drugs, although whether PAKs are required for cancer metastasis is unknown.

Many previous studies characterizing the functions of PAKs have used overexpression of dominant-negative and constitutively active mutants which have a major drawback because

they can sequester adapter proteins, substrates and Rho GTPases depending on the mutations present. Rapid advances in the availability and use of RNAi provides an alternative means to study the function of individual PAK isoforms in cancer cell migration without sequestering proteins from interconnected signalling pathways.

Much previous work on PAK function was also carried out in non-human cells or cells originating from non-cancerous tissue and it is possible that the roles and importance of PAKs could be different in cancer cells. It has recently been shown that the DU145 cancer cell line scatters in response to HGF providing a model system to investigate cell migration and scattering in cancer cells.

The aim of this project is therefore to investigate the roles of PAK isoforms in cancer cells using siRNA and to define isoform-specific roles in cell migration and scattering stimulated by HGF.

Chapter 2: Materials and methods**2.1: Materials****2.1.1 Tissue culture materials**

Tissue culture reagents		
Reagent	Manufacturer	Catalog number
Dulbecco's modified eagle medium (DMEM) (Glucose (4500 mg/L) + L-glutamine (580 mg/L) + Pyruvate (110 mg/L))	Gibco (Invitrogen)	41966-029
Foetal Calf Serum (FCS)	Biosera	S1810
Penicillin/streptomycin (100X)	Gibco (Invitrogen)	
Phosphate buffered saline (PBS)	Gibco (Invitrogen)	14190
Phosphate buffered saline (PBS) +Mg ²⁺ +Ca ²⁺	Gibco (Invitrogen)	14040
Roswell Park Memorial Institute (RPMI) 1640 (+ 300 mg/L L-Glutamine + 25 mM HEPES)	Gibco (Invitrogen)	52400-025
Roswell Park Memorial Institute (RPMI) 1640 (+ 300 mg/L L-Glutamine)	Gibco (Invitrogen)	21875-034
Trypsin/EDTA	Gibco (Invitrogen)	25300

Table 2.1: Reagents used for tissue culture**2.1.2: Protein biochemistry materials**

Protein biochemistry solutions	
Buffer/solution	Composition
Cell lysis buffer	10 mM Tris-Cl pH 7.6 5 mM EDTA 150 mM NaCl 30 mM Na Pyrophosphate 0.5% Triton X-100 Complete mini EDTA-free protease inhibitor. PhosSTOP phosphatase inhibitor
10X SDS-PAGE running buffer	248 mM Tris-Cl 1.92 M Glycine 10% SDS (w/v)
4X SDS sample buffer	0.25 M Tris-Cl pH6.8 8% SDS (w/v) 40 % Glycerol (w/v) 2.82 M β -mercaptoethanol 0.2% Bromophenol blue (w/v)

SDS-PAGE 7.5% gel	375 mM Tris-Cl pH 8.8 7.5% Acrylamide (w/v) 0.1% SDS (w/v) 0.1% APS (w/v) 0.005% TEMED (w/v)
SDS-PAGE stack gel	126 mM Tris-Cl pH 6.8 10% Acrylamide (w/v) 0.1% SDS (w/v) 0.1% APS (w/v) 0.01% TEMED (w/v)
10X SDS-PAGE transfer buffer	248 mM Tris-Cl, 1.92 M Glycine (20% Methanol added upon dilution to 1X)
Tris-buffered saline + 0.1% tween (TBS-T)	50 mM NaCl, 25 mM Tris-Cl pH 7.6 0.1% Tween (w/v)
Immunoprecipitation wash buffer	10 mM Tris-Cl 5 mM EDTA 150 mM NaCl 30 mM Na Pyrophosphate 0.5% Triton X-100 (w/v)

Table 2.2: Composition of solutions used for protein biochemistry.

Protein Biochemistry reagents		
Reagent	Manufacturer	Catalogue number
Complete, mini EDTA-free protease inhibitor cocktail	Roche Applied Science	04 693 159 001
PhosSTOP phosphatase inhibitor cocktail	Roche Applied Science	04 906 837 001
Precision Plus protein all blue standards	BIO-RAD	161-0373EDU
Enhanced chemiluminescence (ECL) reagents	Amersham biosciences	RPN2106V2
PageRuler prestained protein ladder	Fermentas	#SM0671
Dried, skimmed milk	Marvel	N/A
Bovine Serum Albumin (BSA)	Sigma	A7906-100G
MOPS running buffer	Invitrogen	NP0001
Immobilon-P PVDF membrane	Millipore	IPVH00010
Immobilon-FL PVDF membrane	Millipore	IPFL00010

Table 2.3: Reagents used for protein biochemistry.

Protein biochemistry equipment	
Equipment	Manufacturer
NuPAGE 4-12% polyacrylamide gels	Invitrogen
Nu PAGE X-Cell II electrophoresis system	Invitrogen
Mini-Protean electrophoresis and western blotting system	Bio-Rad

Table 2.4: Equipment used for protein biochemistry.

Primary antibodies used on western blots				
Antigen	Species	Dilution	Manufacturer	Catalogue number
β -actin	Mouse	1/4000	Sigma-Aldrich	A-5441
p-Akt (S473)	Rabbit	1/1000	Cell Signaling Technology	#4058
Cdc42	Rabbit	1/1000	Cell Signaling Technology	F-2168
p-cofilin (S3)	Rabbit	1/1000	Cell Signaling Technology	#3311
E-cadherin	Rabbit	1/1000	Transduction Labs	610181
ERK1/2	Rabbit	1/1000	Santa Cruz Biotechnology	sc-94
p-ERK1/2 (T202/Y204)	Rabbit	1/1000	Cell Signaling Technology	#9101L
p-FAK (Y576/577)	Rabbit	1/1000	Cell Signaling Technology	#3281
LIMK1	Rabbit	1/1000	Cell Signaling Technology	#3842
p-LIMK1/2 (T508)	Rabbit	1/1000	Cell Signaling Technology	#3841
p-MEK (S298)	Rabbit	1/1000	Abcam	ab5613-50
p-R-MLC (T18/S19)	Rabbit	1/1000	Cell Signaling Technology	#3674
p-Op18 (S16)	Rabbit	1/1000	Santa Cruz Biotechnology	sc-12948-R
p-p38 (T180/Y182)	Rabbit	1/1000	Cell Signaling Technology	#9211
PAK1	Rabbit	1/1000	Cell Signaling Technology	#2602
PAK2	Rabbit	1/1000	Cell Signaling Technology	#2608
PAK3	Rabbit	1/1000	Cell Signaling Technology	#2609
PAK4	Goat	1/1000	Santa Cruz Biotechnology	sc-18622
PAK4	Rabbit	1/1000	Cell Signaling Technology	#3442
p-PAK1/2/3 (T423)	Rabbit	1/1000	Cell Signaling Technology	#2601
p-PAK1/2/3 (T423)	Rabbit	1/1000	Abcam	ab-2477-100
p-PAK1/2 (S199/204)	Rabbit	1/1000	Cell Signaling Technology	#2605
p-PAK1/2/3 (S141)	Rabbit	1/1000	Biosource	44-940
p-Raf (S338)	Rabbit	1/1000	Cell Signaling Technology	#9427
Rac1	Mouse	1/1000	Upstate	05-389
Secondary antibodies used on western blots				
Antibody	Species	Concentration	Manufacturer	Catalogue number
Horseradish peroxidase-conjugated anti-mouse IgG	Sheep	1/15000	Amersham Biosciences	NA-931
Horseradish peroxidase-conjugated anti-rabbit IgG	Donkey	1/2000	Amersham Biosciences	NA-934

IR DYE 800-linked anti-mouse IgG	Donkey	1/2000	Rockland	610-732-124
Alexa 680-linked anti-rabbit IgG	Goat	1/2000	Molecular Probes	A21076
Alexa 680-linked anti-goat IgG	Donkey	1/2000	Molecular Probes	A21084

Table 2.5: Primary and secondary antibodies used for probing western blots.

2.1.3: Cell biology materials

Cell biology reagents		
Reagent	Manufacturer	Catalogue number
Human recombinant Hepatocyte Growth Factor (HGF)	R and D systems	294-HG-005
Fluorescent mounting medium	Dakocytomation	S3023
20 µl aerosol-resistant tips for scratch assays	Rainin	RT-20F
Matrigel-coated transwells (8 µm pore size)	BD Biosciences	354480
Uncoated transwells (8 µm pore size)	Falcon (BD Biosciences)	355097
Lipofectamine 2000	Invitrogen	11668-019
Optimem GlutaMAX	Gibco (Invitrogen)	51985-026
A/G-linked agarose beads	Santa Cruz Biotechnology	sc-2003
13 mm coverslips	VWR	631-0150
Sterile filtered mineral oil	Sigma	M5310-1L

Table 2.6: Reagents used in cell biology experiments.

short interfering RNA oligomers			
Oligomer	Supplier	Serial number	Sense (top) and antisense (bottom) sequences
siControl_Q	Qiagen	1022076	UUCUCCGAACGUGUCACGdTdT ACGUGACACGUUCGGAGAAdTdT
siControl_D	Dharmacon	D-001810-01	ON-TARGET ^{plus} modified, sequence not available
Cdc42_02	Dharmacon	D-005057-02	GAUUACGACCGCUGAGUUAUU 5' - PUAACUCAGCGGUCGUAUUCUU
Cdc42_03	Dharmacon	D-005057-03	GAUGACCCUCUACUAUUGUU 5' - PCAAUAGUAGAGGGGUCAUCUU
PAK1a	Dharmacon	D-003521-01	GAAGAAUAUACACGGUUUUU 5' - PAAACCGUGUAUAUUUCUUCUU
PAK1b	Dharmacon	D-003521-03	CAUCAAAUAUCACUAAGUCUU

			5' – PGACUUAGUGAUUUUGAUGUU
PAK1c	Dharmacon	D-003521-05	CAACAAAGAACAUCACUAAU 5' – PUAGUGAUUGUUCUUUGUUGUU
PAK1d	Dharmacon	D-003521-07	AGAAAUACCAGCACUAUGAUU 5' – PUCAUAGUGCUGGUAAUUUCUUU
PAK1e	AstraZeneca	N/A; from collaborators	CACACAAUUCAUGUCGGUdTdT AACCGACAUGAAUUGUGUGdTdT
PAK2a	Dharmacon	D-003597-05	AGAAGGAACUGAUCAUUAAUU 5' – PUUAAUGAUCAGUUCUUCUUU
PAK2b	Dharmacon	D-003597-06	CUACAGACCUCCAAUAUCAUU 5' – PUGAUUUUGGAGGUCUGUAGUU
PAK2c	Dharmacon	D-003597-07	GAAACUGGCCAAACCGUUAUU 5' – PUAACGGUUUGGCCAGUUUCUU
PAK2d	Dharmacon	D-003597-09	ACAGUGGGCUCGAUUACUAAU 5' – PUAGUAAUCGAGCCCACUGUUU
PAK4a	Dharmacon	D-003615-03	GGAUAAUGGUGAUUGAGAUUU 5' – PAUCUCAAUCCACCAUUAUCCUU
PAK4b	Dharmacon	D-003615-05	GGGUGAAGCUGUCAGACUUUU 5' – PAAGUCUGACAGCUUCACCCUU
PAK4c	Dharmacon	D-003615-06	AGAAUGUGGUGGAGAUGUAUU 5' – PUACAUCUCCACCACAUUCUUU
PAK4d	Dharmacon	D-003615-07	CCAUGAAGAUGAUUCGGGAUU 5' – PUCCCGAAUCAUCUUAUGGUU
Rac1_05	Dharmacon	D-003560-05	AGACGGAGCUGUAGGUAAAUU 5' – PUUUACCUACAGCUCCGUCUUU
Rac1_07	Dharmacon	D-003560-07	UAAGGAGAUUGGUGCUGUAUU 5' – PUACAGCACCAUUCUCCUUAUU
Rac2_01	Dharmacon	D-007741-01	GAGAUGGGGCCGUGGGCAAUU 5' – PUUGCCCACGGCCCCAUCUCUU
Rac2_02	Dharmacon	D-007741-02	CAGCCAAUGUGAUGGUGGAUU 5' – PUCCACCAUCACAUUGGCUGUU
Rac2_03	Dharmacon	D-007741-03	CCAAUGUGAUGGUGGACAGUU 5' – PCUGUCCACCAUCACAUUGGUU
Rac2_04	Dharmacon	D-007741-04	ACAAGGACACCAUCGAGAAUU 5' – PUUCUCGAUGGUGUCCUUGUUU
Rac3_01	Dharmacon	D-008836-01	GGAGAUUGGCUCUGUGAAAUU 5' – PUUUCACAGAGCCAAUUCUCCUU
Rac3_02	Dharmacon	D-008836-01	ACGUGAUGGUGGACGGGAAUU 5' – PUUCCCGUCCACCAUCACGUUU
Rac3_04	Dharmacon	D-008836-01	CGAGAAUGUUCGUGCCAAGUU 5' – PCUUGGCACGAACAUUCUCGUU
Rac3_05	Dharmacon	D-008836-01	AAACUGACGUCUUUCUGAUUU 5' – PAUCAGAAAGACGUCAGUUUUU
RhoA_11	Dharmacon	J-003860-11	On-Target+ GACCAAAGAUGGAGUGAGAUU 5' – PUCUCACUCCAUCUUUGGUCUU
RhoJ_05	Dharmacon	J-010367-05	ON-TARGET ^{plus} modified, sequence not available
RhoJ_06	Dharmacon	J-010367-06	ON-TARGET ^{plus} modified, sequence not available

			available
RhoJ_07	Dharmacon	J-010367-07	ON-TARGET _{plus} modified, sequence not available
RhoJ_08	Dharmacon	J-010367-08	ON-TARGET _{plus} modified, sequence not available
RhoQ_05	Dharmacon	J-009943-05	ON-TARGET _{plus} modified, sequence not available
RhoQ_06	Dharmacon	J-009943-06	ON-TARGET _{plus} modified, sequence not available
RhoQ_07	Dharmacon	J-009943-07	ON-TARGET _{plus} modified, sequence not available
RhoQ_08	Dharmacon	J-009943-08	ON-TARGET _{plus} modified, sequence not available
RhoU_01	Dharmacon	D-009882-01	GUACUGCUGUUUCGUAUGAUU 5' - PUCAUACGAAACAGCAGUACUU
RhoU_02	Dharmacon	D-009882-02	GAACGUCAGUGAGAAAUGGUU 5' - PCCAUUUCUCACUGACGUUCUU
RhoU_03	Dharmacon	D-009882-03	CAGAGAAGAUGUCAAGUCUU 5' - PGACUUUGACAUCUUCUCUGUU
RhoU_04	Dharmacon	D-009882-04	AAGCAGGACUCCAGAUAAAUU 5' - PUUUAUCUGGAGUCCUGCUUUU
RhoV_01	Dharmacon	D-006374-01	GAGGGACGAUGUCAACGUAUU 5' - PUACGUUGACAUCGUCCUCUU
RhoV_02	Dharmacon	D-006374-02	GAAGAAACUGAAUGCCAAAUU 5' - PUUUGGCAUUCAGUUUCUUCUU
RhoV_03	Dharmacon	D-006374-03	GUUUUGACUCGGCUAUUCUU 5' - PGAAUAGCCGAGUCAAAUACUU
RhoV_04	Dharmacon	D-006374-04	GCUCAGCCUUGACGCAGAAUU 5' - PUUCUGCGUCAAGGCUGAGCUU

Table 2.7: siRNA oligomers used for protein knockdown.

Primary antibodies and fluorescent probes used for immunofluorescence				
Antigen	Species	Concentration	Manufacturer	Catalogue number
F-actin (Phalloidin)- (Alexa 546-linked)	N/A	1/200	Invitrogen	A22283
Tubulin-(FITC-linked)	Mouse	1/200	Sigma	
E-Cadherin	Rabbit	1/100	Transduction Labs	610181
p95PKL (GIT1/2)	Mouse	1/100	Transduction Labs	611388
ERK1/2	Rabbit	1/100	Santa Cruz Biotechnology	sc-94
Hoechst 33342	N/A	1/2000	Invitrogen	H3570
Secondary antibodies used for immunofluorescence				
Antigen	Species	Concentration	Manufacturer	Catalogue number
Alexa 647-linked anti- rabbit IgG	Goat	1/200	Invitrogen	A21245

Alexa 488-linked anti-mouse IgG	Goat	1/200	Invitrogen	A11001
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Table 2.8: Antibodies used for immunofluorescence.

Cell biology software	
Software	Source
Photoshop CS3 software	Adobe
Metamorph software	Molecular Devices
ImageJ	National Institutes of Health
LSM510 software	Zeiss
Definiens imaging software	Definiens

Table 2.9: Software used for image capture, processing and analysis.

Cell biology microscopes/instruments	
Software	Source
LSM510 confocal microscope	Carl Zeiss
Aiovert 135 microscope	Carl Zeiss
Eclipse TE 100-E microscope	Nikon
ArrayScan imaging system	Cellomics

Table 2.10: Microscopes and instruments used for image capture.

Growth Media	
Cell lines	Medium
DU145, PC3 Growth medium	RPMI + 10% FCS, L-glutamine (300 mg/L), penicillin (100 IU/ml) and streptomycin (100 µg/ml), + 25 mM HEPES for experiments or HEPES free for general cell culture.
DU145, PC3 Starvation medium	RPMI + 0.1% FCS, L-glutamine (300 mg/L), penicillin (100 IU/ml) and streptomycin (100 µg/ml), + 25 mM HEPES for experiments or HEPES free for general cell culture.
MCF7, HT29 Growth medium	DMEM + 10% FCS, Glucose (4500 mg/L), L-glutamine (580 mg/L), penicillin (100 IU/ml) and streptomycin (100 µg/ml).
MCF7, HT29 Starvation medium	DMEM + 0.1% FCS, Glucose (4500 mg/L), L-glutamine (580 mg/L), penicillin (100 IU/ml) and streptomycin (100 µg/ml).

Table 2.11: Composition of growth media used for tissue culture and experiments

2.2: Methods

2.2.1: Tissue culture

2.2.1.1: Passaging cell lines

For passaging, cells were washed twice in phosphate buffered saline (PBS) and incubated with 1 ml trypsin/EDTA at 37°C until all cells had detached. Cells were then suspended in 10 ml growth medium and 2ml of this was added to a new flask already containing a further 10 ml growth medium.

All experiments except for Figures 3.1 – 3.4, 4.1 and 4.19 were carried out in medium supplemented with 25 mg/L HEPES

2.2.2: Protein Biochemistry

2.2.2.1: HGF stimulation for cell lysis

Cells were seeded in 24-well plates at the following densities and allowed to adhere for 18 h: DU145, 4×10^5 /well; PC3, 2×10^5 /well; MCF7, 4×10^5 /well; HT29, 8×10^5 /well. If cells were to be transfected, antibiotic-free medium was used. Cells were then either transfected with siRNA according to the protocol (2.2.3.1) or untreated and maintained in growth medium. After a further 48 h, medium was removed and replaced with 1 ml fresh growth medium containing 10 ng/ml HGF. Cells were lysed at the desired time points.

2.2.2.2: Cell lysis

Cells were grown and treated as required. Medium was removed from the cells and they were washed once with PBS +Mg²⁺ +Ca²⁺. Cells were then placed on ice and an appropriate volume of TritonX-100 lysis buffer was added (in 24-well plates, 100 µl lysis buffer was used per well). Following incubation on ice for 5 min, cells were scraped from the substrate using a wide-bore pipette tip, transferred to a microfuge tube and incubated on ice for 10 min. Lysates were then centrifuged at 4°C and 16.2 G (Thermo Scientific Fresco 17 centrifuge) for 10 min and the supernatant was transferred to a fresh microfuge tube. For figures 3.2, 3.3 and 3.4, protein concentration was assayed using the BioRad protein assay protocol shown below. 4X sample buffer was added to each supernatant, heated to 100°C for 5 min and stored at -20°C.

2.2.2.3: BioRad protein concentration assay

5 µl of cell lysate (or fresh lysis buffer as a blank) was added to a plastic cuvette containing 795 µl ddH₂O and 200 µl BioRad protein assay reagent. Solutions were mixed by inversion several times and absorbance was measured at 595 nm using a BioRad spectrophotometer.

2.2.2.4: Immunoprecipitation

DU145 cells were seeded in 6 cm dishes at a density of 5×10^5 cells/dish. After 32 h, medium was removed and replaced with fresh growth medium or starvation medium containing 0.1% FCS. After a further 16 h, cells were sequentially stimulated with fresh starvation medium containing 10 ng/ml HGF over a time course of 2 h. Cells were then lysed in 1 ml Triton X-100 lysis buffer according to the protocol above except that sample buffer was not added. Lysates were pre-cleared by rotation with 5 µl protein A/G-linked agarose beads for 30 min at 4°C followed by centrifugation at 6000 G for 5 min. The supernatant was transferred to a fresh tube and immunoprecipitation was performed; for each lysate, 20 µl protein A/G-linked agarose beads were combined with 2.5 µl PAK4 antibody (Cell Signaling Technology). A further two tubes containing 20 µl protein A/G-linked agarose beads and 2.5 µl Myc antibody were prepared as a control for non-specific binding. 0.5 ml ice-cold PBS was added to each tube and rotated at 4°C for 2 h. Beads were centrifuged at 6000 G. for 5 min, supernatant was removed and beads were washed twice in lysis buffer. 10 µl of 10% BSA was added to each sample of beads and 500 µl pre-cleared lysate was transferred to each. (Myc-linked beads received 500 µl lysate from starved, unstimulated and starved cells stimulated with HGF for 60 min) Lysates/beads were rotated at 4°C for 2 h then centrifuged for 5 min at 6000 G. Supernatant was removed and beads were washed 4 times in wash buffer. Finally, supernatant was removed, 15 µl 2X sample buffer was added to each tube and heated to 100°C for 5 min Samples were stored at -20°C prior to western blotting.

2.2.2.5: NuPAGE (SDS-PAGE using pre-cast gels)

An X-Cell II gel tank was assembled using a pre-cast SDS-PAGE gel of the appropriate concentration and composition (usually 4-12% polyacrylamide, 1.5 mm thickness). Lysates in sample buffer were added to the wells and Bio-Rad precision plus all-blue standard was

used as molecular weight markers. Where protein concentration was measured, an equal amount of protein was loaded into each lane. For other lysates, an equal volume of sample was loaded. Electrophoresis was carried out at 120 volts for approximately 1.5 h and the resulting gel was used for western blotting (2.2.2.7).

2.2.2.6: SDS-PAGE using non-precast gels

When pre-cast gels were not used, proteins were separated by electrophoresis through a 7.5%, 1.5 mm polyacrylamide gel using a Mini-Protean electrophoresis system. The gel mixture was prepared, poured into the scaffold and overlaid with water-saturated butanol. After 30 min, the butanol was washed off, a 15-well or 10-well comb was inserted and the stacking gel mixture was prepared and pipetted on top of the gel. Once set, the gel was placed in a Mini-Protean gel-tank which was filled with 1X SDS-PAGE running buffer. Lysates in sample buffer were added to the wells and PageRuler prestained protein ladder was used as molecular weight markers. Where protein concentration was measured, an equal amount of protein was loaded into each lane. For other lysates, an equal volume of sample was loaded. Electrophoresis was carried out at 100 volts for approximately 1.5 h. The resulting gel was used for western blotting (2.2.2.7).

2.2.2.7: Western blotting

Following SDS-PAGE, proteins were transferred onto polyvinylidene fluoride (PVDF) membrane (Immobilon-FL, or Immobilon-P) by wet transfer using the Mini Protean transfer system (Bio-Rad). Sponges and Whatmann paper were pre-soaked in 1X transfer buffer, and PVDF was pre-soaked in methanol. The polyacrylamide gel was overlaid with PVDF and sandwiched between two pieces of blotting paper and two sponges in a Mini Protean transfer cassette. The cassette was inserted into a transfer tank containing 1X transfer buffer at 4°C and electrophoresed at 80 V for 90 min. The resulting membrane with bound proteins was blocked for 1 h in TBS-T containing 5% BSA. Blocking buffer was then replaced with TBS-T containing 5% BSA or 5% milk and the appropriate primary antibody and incubated overnight at 4°C (or for 1 h at room temperature for β -actin). The membrane was then washed four times in TBS-T for 5 min and incubated with HRP-linked or IR dye-linked secondary antibody at a concentration of 1:4000 in TBS-T + 1% Milk for 1 h. The membrane was washed in TBS-T briefly twice, and then again four times for 5 min and developed by addition of ECL reagent and exposure to X-ray film (for HRP-linked

secondary antibodies) or imaged using the LI-COR Odyssey IR imaging system (For IR dye-linked secondary antibodies).

2.2.2.8: Quantification of western blots

Films were scanned and resulting images were analysed using ImageJ software. First the image was inverted to give a positive grey value for light pixels and a selection box was placed over the first band. The average grey value was recorded and the selection box was moved to the next band so the selection area remained the same for each. A grey value for the background was measured and subtracted from the band measurements. If a final normalized grey value was less than 0 (less than background) it was counted as 0.

2.2.3: Cell Biology

2.2.3.1: Transfection of cells with siRNA using Lipofectamine 2000

In experiments where cells were transfected, antibiotic-free media were used throughout the protocols. Cells were seeded, allowed to adhere for at least 7 h and then given fresh growth medium (500ul in 24-well plates, 2.5 ml in 2 cm dishes or SlideFlasks) before siRNA treatment. Lipofectamine 2000 (Invitrogen) was diluted in Optimem (Invitrogen) in a ratio of 1:50 and incubated at room temperature for 5 min (Buffer B). siRNA was diluted to a concentration of 40 pmoles/ml in Optimem Glutamax and an equal volume of Buffer B was added. This mixture was incubated at room temperature for 20 min and then added to the cells (100 µl (2 pmoles siRNA) in 24-well plates or 500 µl (10 pmoles siRNA) in 2 cm dishes and SlideFlasks. Medium was removed after between 5 and 24 h and replaced with 1ml fresh antibiotic-free medium.

2.2.3.2: Scattering and random migration assays

Cells were seeded at the following densities and allowed to adhere for 18 h: DU145, 1.2×10^5 cells/well in a 24-well plate (or 6×10^5 cells/2 cm dish for Figure 4.1); PC3, 8×10^4 cells/well in a 24-well plate (or 4×10^5 cells/2 cm dish for Figure 4.1); MCF7, 6×10^5 cells/2 cm dish; HT29 cells 1×10^6 cells/ 2 cm dish.

For Figure 4.1, cells were maintained in growing conditions for a total of 48 h prior to starvation in medium containing 0.1% FCS for 6 h. Cells were then stimulated with 10 ng/ml HGF and movies were made for 16 h using an Axiovert 135 microscope.

For Figure 4.2 cells were maintained in growing conditions for a total of 48 h then either starved in medium containing 0.1% FCS or kept in growing conditions for 6 h. Medium was then changed and cells were either kept unstimulated in 0.1% FCS or stimulated with 10 ng/ml or 20 ng/ml HGF. Mineral oil was placed on top of the medium to maintain pH and movies were made for 24 h.

For all other scattering or random migration assays, cells were either transfected with siRNA using Lipofectamine 2000 or untreated and maintained in growth medium as required. 54 h after transfection, medium was removed and replaced with fresh growth medium or growth medium supplemented with 10 ng/ml HGF. Mineral oil was placed on top of the medium to maintain pH and time lapse movies were made over a period of up to 24 h using a Nikon TE2000-E microscope with a Plan Fluor 10X objective. Image series were captured using Metamorph software.

2.2.3.3: Analysis of cell rounding

Appropriate frames from movies of DU145 cell scattering were analysed (Figure 2.1A). Image brightness and contrast were adjusted using Adobe Photoshop CS3 software; brightness -50, contrast +100 with 'use legacy' ticked. This gave an image where rounded cells had a saturated white circle encompassing a black spot at the nucleus (Figure 2.1B, red arrow). The Paint Bucket tool was used to fill the black background with white leaving any discontinuous spots, i.e. nuclei of rounded cells, black (Figure 2.1C). The brightness and contrast used defined the stringency of counting to ensure that no flat cells were counted as round cells. Metamorph software was used to automatically count the number of black objects in each image. Size exclusions were set to include spots corresponding to whole cells but not smaller background objects.



Figure 2.1: Processing of images using Adobe Photoshop CS3 for cell rounding measurements. As described, frames from movies (A) were extracted from image sequences and brightness/contrast was altered to make an image with saturated phase-bright objects in white (B). The background was filled with white but any discontinuous black objects i.e. the nuclei of rounded cells remained black (C). Black objects of appropriate size were counted using Metamorph.

2.2.3.4: Analysis of lamellipodium length

After DU145 cell scattering assays for 24 h, the final frame from movies was analysed. Cells not in contact with more than one other cell which had at least one lamellipodium were selected for measurement so that round cells with no lamellipodia, or cells still within groups did not influence the data. Lamellipodium length was measured using ImageJ software by drawing a line along the leading edge of each lamellipodium and making a measurement in pixels.

2.2.3.5: DU145 cell scratch assays using the Cellomics ArrayScan instrument

DU145 cells were seeded at a density of 8000 cells/well in black-sided 96-well plates. After 24 h, medium was removed and replaced with 83 μ l fresh medium before siRNA treatment. Cells were transfected with siRNA using Lipofectamine 2000 or left untransfected. (Note that siRNA was not washed off after 4 h). 48 h after transfection medium was removed from each well, cells were washed once and medium was replaced with either fresh growth medium or starvation medium containing 0.1% FCS. After a further 7 h a 200 μ l pipette tip was used to scratch the monolayer in the centre of each well, medium was then removed and replaced with fresh medium or medium containing 10 ng/ml HGF. Cells were then incubated for 16 h prior to fixation in 3.7% formaldehyde and staining for F-actin (Phalloidin) and nuclei (Hoechst).

The centre of each well was imaged automatically using the Cellomics ArrayScan system and the Definiens program was used with an algorithm written by collaborators at AstraZeneca to measure the area of each scratch based on the phalloidin stain. Data from wells where the scratch was off-centre and not imaged completely were not included in subsequent data analysis.

2.2.3.6: DU145 cell scratch assays using time-lapse microscopy

DU145 cells were seeded at 7.5×10^5 cells/well in a 24-well plate and allowed to adhere and grow for 16 h. Cells were transfected with siRNA or left untreated in growth medium. 48 h after transfection, medium was removed from each well and replaced with starvation medium containing 0.1% FCS. After a further 8 h, when cells had formed a confluent monolayer, a 20 μ l pipette tip was used to scratch a line of cells from the centre of the well. Medium was then removed and replaced with fresh starvation medium or starvation medium supplemented with 10 ng/ml HGF and time-lapse movies were made of scratches healing over a period of 16 h.

Frames from movies of DU145 cell scratch assays were taken and Adobe Photoshop CS was used to colour the cells black and the scratch white. Metamorph software was used to measure the area covered by white pixels in the resulting images.

2.2.3.7: DU145 Matrigel invasion assays

DU145 cells were seeded in 6-well plates at a density of 1.5×10^5 cells/well and allowed to adhere for 18 h. Cells were transfected with siRNA using Lipofectamine 2000 and kept growing for a further 48 h. Matrigel-coated transwells were rehydrated for 2 h in growth medium at 37°C. Transfected cells were washed twice in PBS, trypsinized and counted. 1×10^6 cells were seeded into the upper chamber of the Matrigel-coated transwells in antibiotic-free growth medium. Growth medium containing 40 ng/ml HGF was placed in the bottom chamber as a chemoattractant. 2×10^5 cells were seeded in uncoated transwells as a migration control with the same chemoattractant concentration. Cells were allowed to invade or migrate for 24 h then remaining cells and matrix were removed from the upper chamber, and cells on the bottom of the transwells were fixed in methanol containing 0.1% crystal violet. 5 separate bright-field images were captured of each transwell with Metamorph software using a Nikon TE2000-E microscope with a Plan Fluor10X objective.

The levels of each image were auto-adjusted to remove background using Adobe Photoshop CS3 autolevels with clipping set to; shadows 9%; highlights 0.1%. Images were then thresholded so that the area of stained cells could be measured automatically using Metamorph software.

2.2.3.8: Acid washing coverslips

13 mm glass coverslips were washed in concentrated nitric acid for 5 min followed by 10 washes with copious amounts of tap water, 5 washes with distilled water and 2 washes with 100% methanol. Coverslips were then baked at 180 °C for 4 h.

2.2.3.9: Immunofluorescent staining

Cells were seeded on acid-washed coverslips in 24-well plates at densities of 1.2×10^5 cells/well (DU145) or 8×10^4 cells/well (PC3). Cells were allowed to adhere for 18 h prior to transfection with siRNA using Lipofectamine 2000. 48 h after transfection, medium was removed and replaced with either fresh growth medium or growth medium containing 10 ng/ml HGF. After a further 24 h, cells were fixed in 4% paraformaldehyde (PFA) for 20 min at room temperature or in methanol at -20 °C for 5 min. Fixed cells were washed three times in PBS and then PFA-fixed cells were permeabilized in 0.1% Triton X-100 for 5 min prior to washing three times again in PBS. All coverslips were then transferred to 5% BSA in PBS.

Primary antibodies were diluted in 5% BSA in PBS and 20 µl spots were pipetted onto parafilm in a 10 cm dish containing water-soaked paper towel to humidify the dish. Coverslips were inverted onto the antibody spots and incubated at 4 °C for 18-22 h.

Secondary antibodies, or phalloidin solutions were prepared in the same way as primary antibodies and coverslips were washed four times in PBS prior to being inverted onto the secondary antibody spots and incubated for 1-3 h at room temperature.

Coverslips were then washed 5 times and mounted onto glass slides using fluorescence mounting medium. Slides were stored in the dark at room temperature for 3-5 h for mounting medium to set and then stored at 4 °C until images were taken.

2.2.3.10: Confocal microscopy

A Zeiss LSM510 confocal laser-scanning microscope with an EC Plan-Neofluar 40X/1.3 oil DIC M27 objective and LSM510 software were used to capture images of fluorescently stained cells with the pinhole set for an approximate optical slice thickness of 1 μm . Images captured were 1056x1056 pixels with a pixel scanning time of 2.18 μs . Microscope and filter settings used are shown:

Fluorophore wavelengths, lasers and filters used				
Fluorophore	Laser	Excitation (nm)	Emission (nm)	Filter (nm)
FITC/Alexa 488	Argon	488/494	520/517	Band Pass 505-530
Alexa 546	Helium/Neon	556	573	Band Pass 560-615 IR
Alexa 647	Helium/Neon	650	668	Meta detector 636-754

Table 2.12: Lasers and filters used for confocal microscopy.

Chapter 3: PAK expression and regulation in cancer cell migration**3.1: Introduction**

PAKs are not uniformly expressed in all tissues and PAK expression can be upregulated in cancer cells (reviewed in Arias-romero et al., 2008 and Kumar et al., 2006). For example, PAK4 mRNA expression is upregulated in multiple breast cancer cell lines (Callow et al., 2002). As PAKs play multiple roles in cell migration, their upregulation in cancer is suggestive of a role in cancer metastasis.

In vitro the cellular processes involved in cancer metastasis including junctional breakdown, cell migration and matrix invasion, can be studied using hepatocyte growth factor (HGF) as a stimulus (Wells et al., 2005 and our unpublished data). HGF has been shown to activate PAK1 and PAK4 in MDCK cells (Royal et al., 2000; Wells et al., 2002). If the same is true in cancer cells it would further implicate a role for PAKs in cancer metastasis.

PAKs are regulated by phosphorylation at multiple sites which affect persistence and level of activation, or binding of adaptor proteins (Chong et al., 2001). In PAK1, phosphorylation of Thr423 in the kinase domain is required for full kinase activity (Zenke et al., 1999). The other group I PAK isoforms contain a highly homologous site (T402 in PAK2 and T421 in PAK3). Group II PAKs contain a homologous site at S474, S602 or S560 in PAK4, PAK5 and PAK6 respectively. Group I PAKs also contain autophosphorylation sites at S199 and S204 in PAK1 or S192 and S197 in PAK2 (Manser et al., 1997). Phosphorylation of these sites follows PAK activation and modulates binding of the adapter protein β -PIX (Mott et al., 2005). It is also possible that these sites are phosphorylated by another kinase or PAK isoform but this has not been described.

Previous studies of PAK isoforms using overexpression of dominant negative and active mutants suggest that PAKs are controlling elements of cell migration and MAP kinase signalling (Kiosses et al., 1999; Qu et al., 2001; Royal et al., 2000; Zhang et al., 1995). However, dominant negative and constitutively active mutants are still able to bind substrate and adapter proteins, thereby sequestering those proteins and blocking multiple signalling pathways. This is highlighted by the finding that dominant negative and constitutively active PAK mutants both inhibit migration of HMEC-1 cells due to the

presence of the N-terminal SH3 binding region in both mutants (Kiosses et al., 1999). Overexpression also makes it difficult to study individual PAK isoforms due to the possibility of shared binding partners, whereby overexpression of one PAK isoform would also inhibit signalling through another. RNA interference (RNAi) provides an alternative to expression of dominant negative mutants by selectively knocking down expression of a target protein. This technique has recently been used to investigate the functions of PAK isoforms, highlighting roles in the breakdown of cell junctions and matrix invasion (Lozano et al., 2008; Pavey et al., 2006). The specificity of RNAi makes it an ideal tool to investigate isoform-specific functions of PAKs.

The aims of this chapter are to investigate how the expression and phosphorylation of PAK1, PAK2 and PAK4 are regulated in cancer cells and to establish a protocol for the knockdown of PAK expression using RNAi.

Results

3.2: PAK expression in cancer cell lines

PAK expression can be altered in cancer compared to normal tissue and upregulation is thought to correlate with progression of the disease. DU145 (prostate), PC3 (prostate), MCF7 (breast) and HT29 (colon) cancer cell lines were chosen for study due to their different morphology and invasion characteristics (Figure 3.1). DU145 cells grow in groups and scatter in response to HGF (Miura 2001). They are less invasive than the other prostate cancer cell line, PC3 cells, which grow as single cells (Colella et al., 2004). MCF7 cells are weakly invasive and junctional breakdown can be stimulated by overexpression of the cell adhesion molecule L1 (Milde-Langosch, 2008; Schtutman, 2006). HT29 cells also grow in groups and respond to HGF stimulation by spreading (Nabeshima 1998).

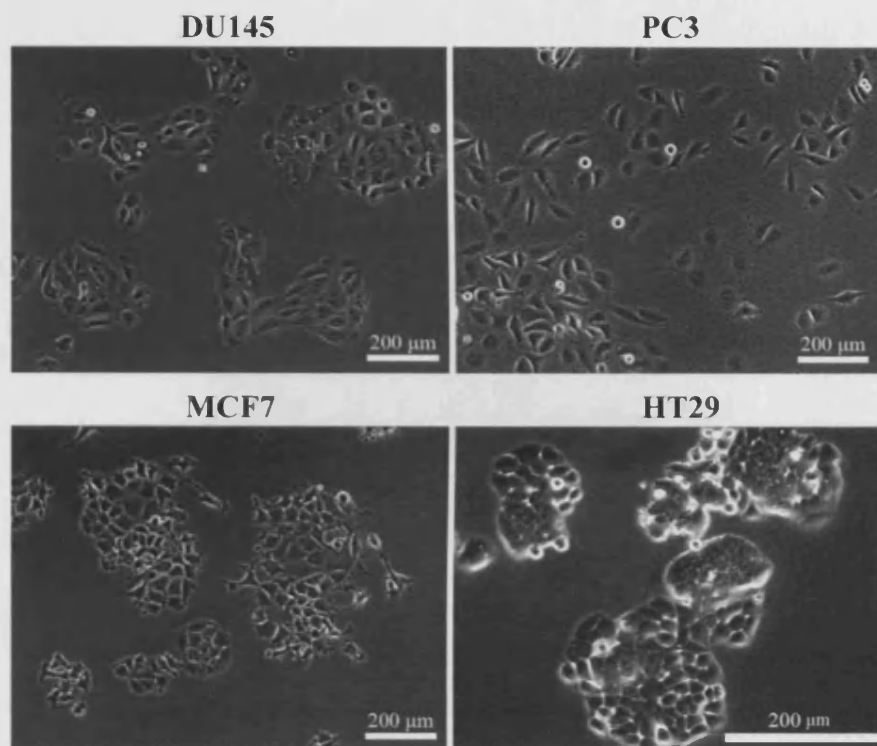


Figure 3.1: Morphology of DU145, PC3, MCF7 and HT29 cells. Cells were seeded and allowed to grow for 48 hours before phase contrast images were captured using a Nikon microscope.

Expression of PAK1, PAK2 and PAK4 was determined by western blotting (Figure 3.2). Each of the cell lines tested expressed PAK1, PAK2 and PAK4 although expression of PAK1 was lower in PC3 and MCF7 cells. PAK4 expression was lowest in DU145 and PC3 cells. An antibody against PAK3 was also tested but gave no signal at the correct molecular weight (data not shown). PAK5 and PAK6 were not tested; PAK5 is thought to be functionally redundant as the PAK5 knockout mouse has no detectable phenotype compared to control mice. PAK6 has been shown to inhibit androgen receptor signalling (Schrantz et al., 2004) but a role in cell migration has not yet been described.

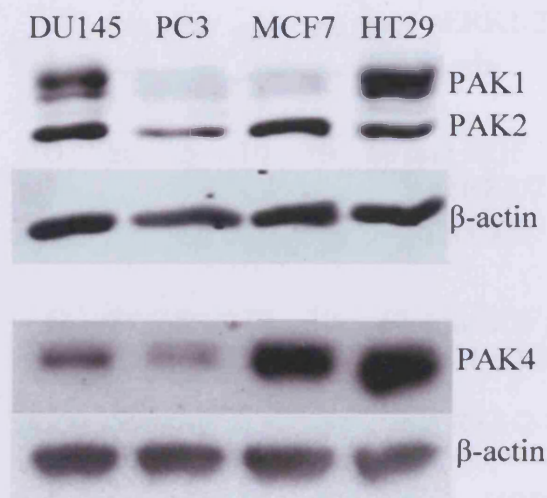


Figure 3.2: Cancer cell lines express PAK1, PAK2 and PAK4. Western blots were performed on cell lysates from DU145, PC3, MCF7 and HT29 cells and probed with antibodies against PAK1, PAK2, PAK4 and β -actin.

3.3: DU145, PC3, MCF7 and HT29 cells respond to HGF

HGF is a growth factor responsible for stimulating cancer cell scattering, increased migration and invasion. PAK1 and PAK4 have been shown to be activated downstream of HGF in MDCK cells (Royal et al., 2000; Wells et al., 2002) but this has not yet been shown in cancer cells. DU145, PC3, MCF7 and HT29 cells express the HGF receptor c-Met (Hiscox and Jiang, 1999; Wells et al., 2005; Wielenga et al., 2000) so provide potential models for studying HGF-induced PAK activation. HGF induces a robust phosphorylation of ERK1/2 so this was used as a readout of HGF signalling. ERK1/2 phosphorylation was observed in DU145, PC3, MCF7 and HT29 cells following stimulation with 10 ng/ml HGF (Figure 3.3).

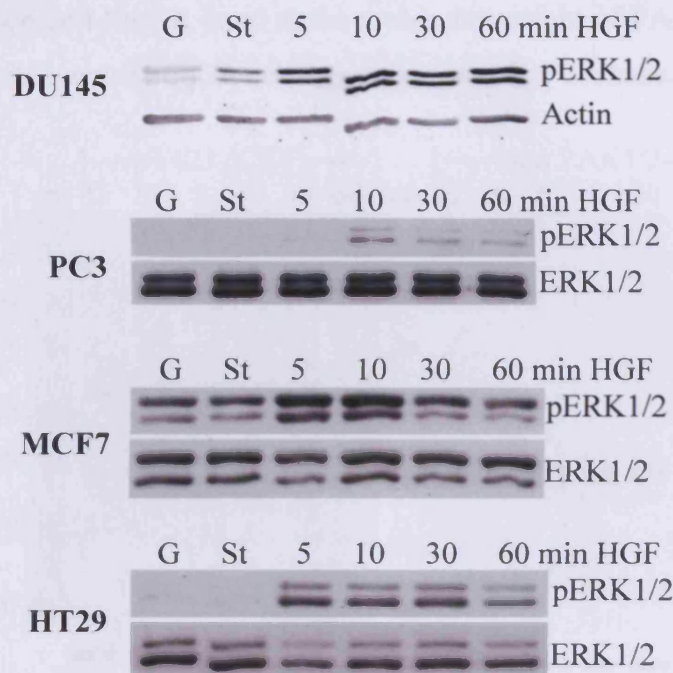


Figure 3.3: HGF induces ERK phosphorylation in the cancer cell lines tested. DU145, PC3, MCF7 or HT29 cells were seeded and allowed to grow for 48 hours. Cells were then either kept in growth medium (G) or serum starved in 0.1% FCS for 16 hours (St) then stimulated with 10 ng/ml HGF for the times shown. Lysates were prepared, western blots were performed and probed with antibodies against phospho-ERK1/2, ERK1/2 or β -actin.

3.4: PAK1 and PAK2 are phosphorylated in response to HGF

Full activation of group 1 PAKs requires phosphorylation of a site equivalent to T423 in PAK1, termed the activation loop (Chapter 1, Section 1.7.1.1). Phosphorylation of this site can be used as a readout of PAK activity. To determine whether HGF stimulates an increase in the activity of PAK1 or PAK2, western blots were probed with an antibody described to recognise PAK1, PAK2 and PAK3 phosphorylated at T423, T402 and T421 respectively (Cell Signaling Technology (CST)) (Figure 3.4). From this point on phosphorylation at this site will be referred to with the PAK1 numbering. The peptide used to generate the antibody is identical to the region in the activation loop of PAK1, PAK2 and PAK3. Results were not conclusive because multiple non-specific bands were observed which are expected to correspond to phosphorylation of Ste20-related kinases as well as other proteins (discussed in detail later). The non-specific bands were observed at a range of molecular weights as shown on the full western blot (Figure 3.4, DU145). However, a band close to the predicted molecular weight of PAK1 increased in intensity with HGF

stimulation in each cell line. A band at the molecular weight of PAK2 was also present in DU145 cells but its intensity did not change following HGF stimulation (Figure 3.4).

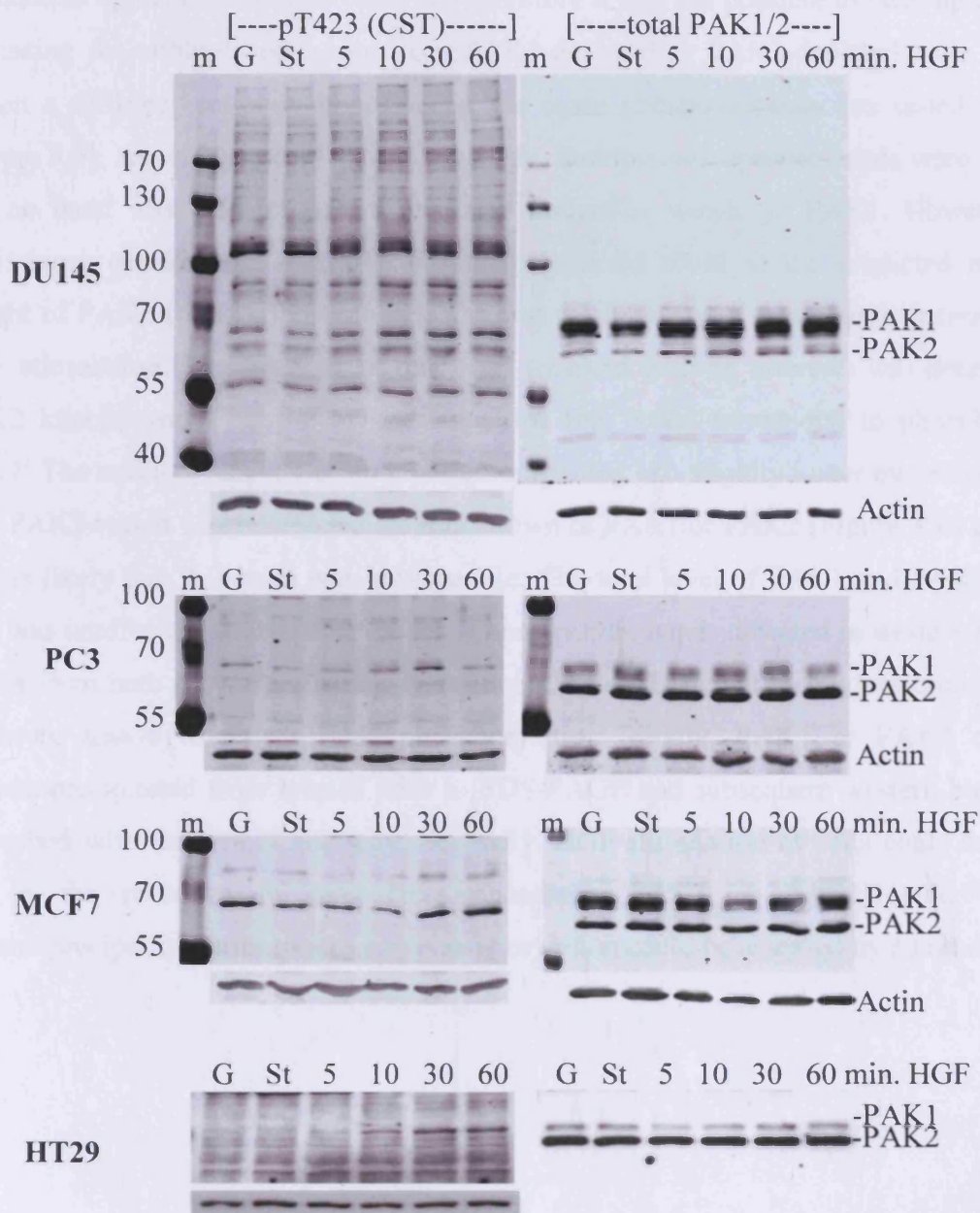


Figure 3.4: Detection of PAK phosphorylation using the pT423 antibody (CST). DU145, PC3, MCF7 or HT29 cells were seeded and allowed to grow for 48 hours. Cells were then either maintained in growth medium (G) or serum starved in 0.1% FCS overnight (St) then stimulated with 10 ng/ml HGF for the times shown. Lysates were prepared, western blots were performed and probed with antibodies against pT423 (Cell Signaling), PAK1, PAK2, actin or ERK1/2. m = marker (kDa), data are from one experiment for DU145 and HT29 cells, and are representative of two experiments for PC3 and MCF7 cells. For HT29, the same lysates were used as for Figure 3.3.

The result with the CST pT423 antibody was reproduced twice in PC3 and MCF7 cells but it can only be considered as a preliminary result as when a new batch of antibody was used, the band at 65 kDa was not detected and therefore it was not possible to back up the result by testing the antibody on lysate from PAK1-depleted or PAK2-depleted cells. For this reason a different antibody raised using the same phosphopeptide was tested (Abcam) (Figure 3.5). As with the CST pT423 antibody, multiple non-specific bands were observed and no band was detected at the predicted molecular weight of PAK1. However, in a preliminary experiment, several bands were detected close to the predicted molecular weight of PAK2 (62 kDa) in each cell line and the intensity of these bands increased with HGF stimulation. The intensity of one band (marked with an asterisk) was decreased by PAK2 knockdown (Figure 3.11) showing that this could correspond to phosphorylated PAK2. The antibody also detected a protein migrating at a slightly lower molecular weight than PAK2 which was unaffected by knockdown of PAK1 or PAK2 (Figure 3.10 and 3.11) so it is likely that this band was non-specific. The total level of PAK1 and PAK2 in each case was unaffected. Due to the number of non-specific bands detected in western blots, the results from both pT423 antibodies were inconclusive. Different techniques could be used in future assays to assess PAK phosphorylation. Firstly, PAK1 or PAK2 could be immunoprecipitated from lysates prior to SDS-PAGE and subsequent western blots could be probed with the pT423 antibody. Secondly, HGF stimulation of cells could be carried out in the presence of radioactive phosphate, PAK1 or PAK2 could then be immunoprecipitated from lysates and phosphorylation could be assessed by autoradiograph.

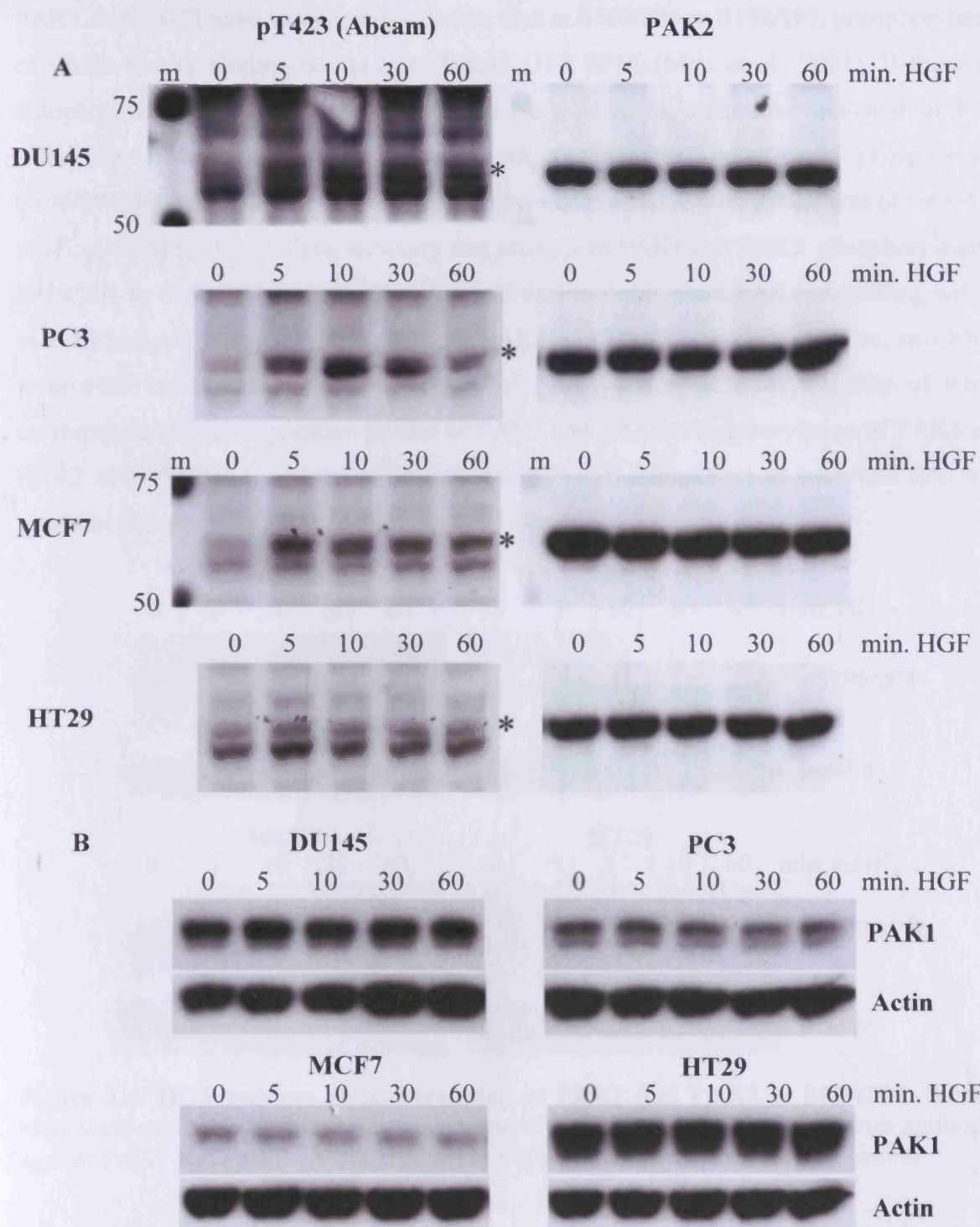


Figure 3.5: Effect of HGF on bands detected by an alternative phospho-T423 antibody (Abcam). Cancer cell lines were stimulated with 10 ng/ml (DU145, PC3, MCF7) or 40 ng/ml (HT29) HGF and lysates were prepared from 0 – 60 minutes after stimulation. Western blots were performed and probed with antibodies against A) pT423 (Abcam) and PAK2 or B) PAK1 and β -actin. m = molecular weight markers (kDa). Asterisks denote the band which was decreased by PAK2 knockdown (Figure 3.11). Data is from one experiment.

PAK1 and PAK2 have autophosphorylation sites at S199/204 or S192/197, phosphorylation of which blocks binding of the Rac1/Cdc42 GEF β PIX (Mott et al., 2005). If these are autophosphorylation sites only, they could be used as an alternative indicator of PAK activation. However, it cannot be ruled out that another kinase could phosphorylate S199/204. Phosphorylation of these sites downstream of HGF stimulation was observed by probing western blots with an antibody that recognises PAK1 and PAK2 phosphorylated at S199/204 or S192/197 respectively (Figure 3.6). From this point PAK1 numbering will be used to indicate phosphorylation of both PAK1 and PAK2. In each cell line, two bands were observed at the molecular weights of PAK1 and PAK2, the intensity of which corresponded to the expression profile of PAK1 and PAK2. Phosphorylation of PAK1 and PAK2 at S199/204 increased rapidly following HGF stimulation in each cell line with maximal phosphorylation at five minutes or before.

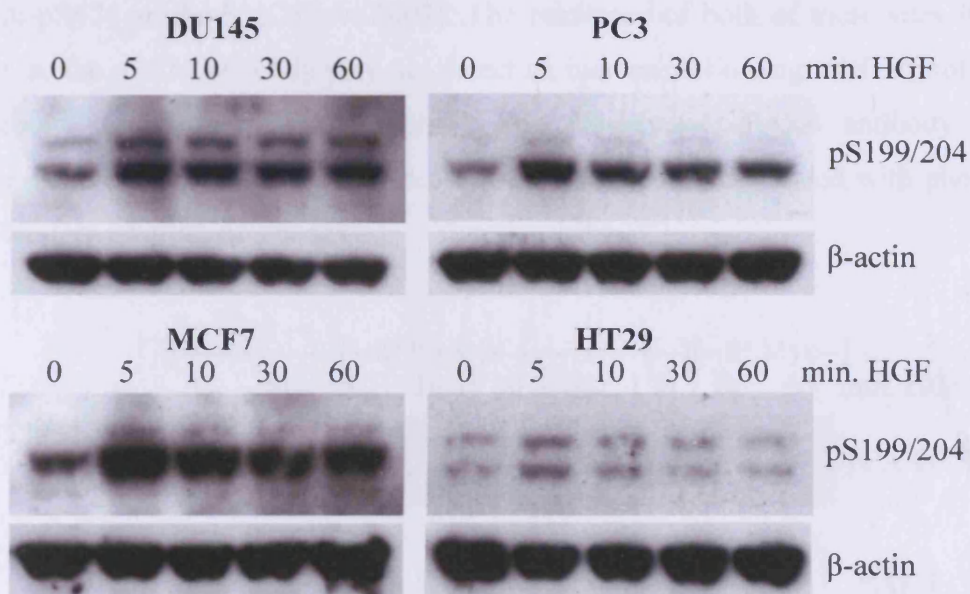


Figure 3.6: HGF induces phosphorylation of PAK1 and PAK2 at S199/204. Western blots were performed using the lysates prepared for Figure 3.7 and probed with antibodies against PAK1 and PAK2 phosphorylated at S199/204 and actin as a loading control.

It has been suggested that group II PAKs also contain an activating autophosphorylation site in the activation loop (Callow 2002) although it is serine rather than threonine; S474 in PAK4; S602 in PAK5 or S560 in PAK6. DU145, PC3, MCF7 and HT29 cells express PAK4 (Figure 3.1) and it has been previously shown that PAK4 is activated downstream of HGF in MDCK cells (Wells et al., 2002). To investigate whether PAK4 or another Group II PAK is phosphorylated at S474 following HGF stimulation of cancer cell lines, an antibody

described to be specific for phosphorylated PAK4, PAK5 and PAK6 was used in PC3 cells but multiple non-specific bands, and no significant change in band intensity following HGF stimulation, meant it was not possible to determine whether PAK4, PAK5 or PAK6 became phosphorylated (result not shown). In order to overcome this and observe the phosphorylation state of PAK4, PAK4 was immunoprecipitated from lysates of cells treated with HGF for 0-60 minutes prior to western blotting (Figure 3.7). The antibody against phosphorylated PAK4, PAK5 and PAK6 did not detect a change in the level of phosphorylated PAK4 following HGF stimulation. However, this may not be indicative of PAK4 activation. It has been shown that S474 is an autophosphorylation site *in vitro* and PAK4 with an S474E mutation is more active than wild type PAK4, but not as active as the PAK4 kinase domain alone suggesting that phosphorylation at S474 is not necessary for kinase activity. There is also a possible phosphorylation site at T478 which blocks binding of an anti-pS474 antibody (Callow 2002). The relevance of both of these sites *in vivo* is unknown so the pS474 antibody may not detect an increase following HGF stimulation but PAK4 could still be activated. To check that the phospho-PAK4 antibody detected phosphorylated and not unphosphorylated PAK4, lysate could be treated with phosphatase prior to western blotting as a control.

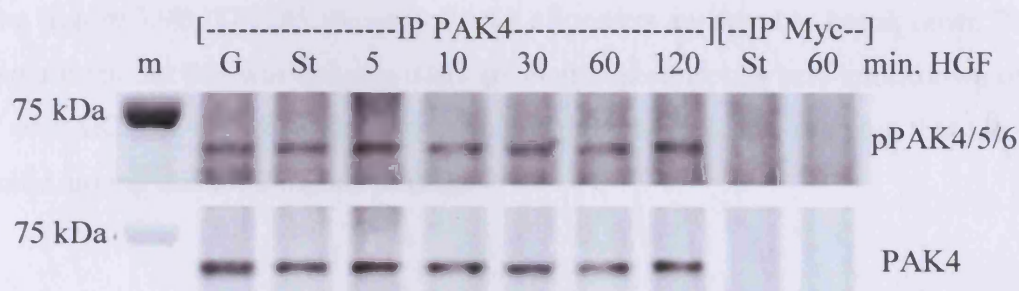


Figure 3.7: An antibody against phosphorylated PAK4 did not detect a change downstream of HGF. PC3 cells were kept in growth medium (G) or starved in 0.1% FCS for 16 hours (St) and stimulated with 10 ng/ml HGF. Cell lysates were prepared at the indicated time points after HGF addition. PAK4 was purified by immunoprecipitation and a western blot of the resulting lysates was probed with an antibody against phosphorylated PAK4/5/6 followed by antibody against total PAK4. An antibody against Myc was used as a negative control for non-specific binding in the immunoprecipitation.

3.5: Individual PAK isoforms can be knocked down in cancer cell lines

The phosphorylation of PAK1 and PAK2 downstream of HGF in cancer cell lines tested suggests that they could play a role in HGF-induced scattering and migration. To investigate this but avoid the problems associated with overexpression of dominant negative and active mutants, RNAi was used to knock down expression of PAK1, PAK2 and also PAK4 in cancer cells. In order to limit off-target effects of RNAi, oligomers should be used at as low a concentration as possible. To determine an appropriate concentration, siRNA against PAK1 was transfected into DU145 cells at varying concentrations and PAK expression was observed by western blotting (Figure 3.8). The most efficient PAK1 knockdown was achieved at a concentration of 1 pmole/well in 24-well plate format (1.7 nM) or above and the level of knockdown was persistent up to 72 hours post-transfection. Later time points were not tested. To be sure of efficient knockdown while limiting the risk of off-target effects, a concentration of 2 pmoles/well or 3.3 nM was chosen for future experiments. To validate results, sets of four separate oligomers of siRNA against PAK1 or PAK2 were tested in DU145, PC3, MCF7 and HT29 cells (Figure 3.9A). Knockdown was achieved in each cell line although PAK1 knockdown in HT29 cells was inefficient. This is possibly due to increased stability of PAK1 protein in this cell line as PAK2 knockdown was successful. PAK4 siRNA was also tested in each cell line (figure 3.9B (DU145 shown)). PAK1 oligomers appeared to knock down PAK2 in one experiment but this was not consistent in other experiments where knockdown of either PAK1 or PAK2 did not affect the expression of the other isoform showing that the siRNA is specific among the homologous proteins.

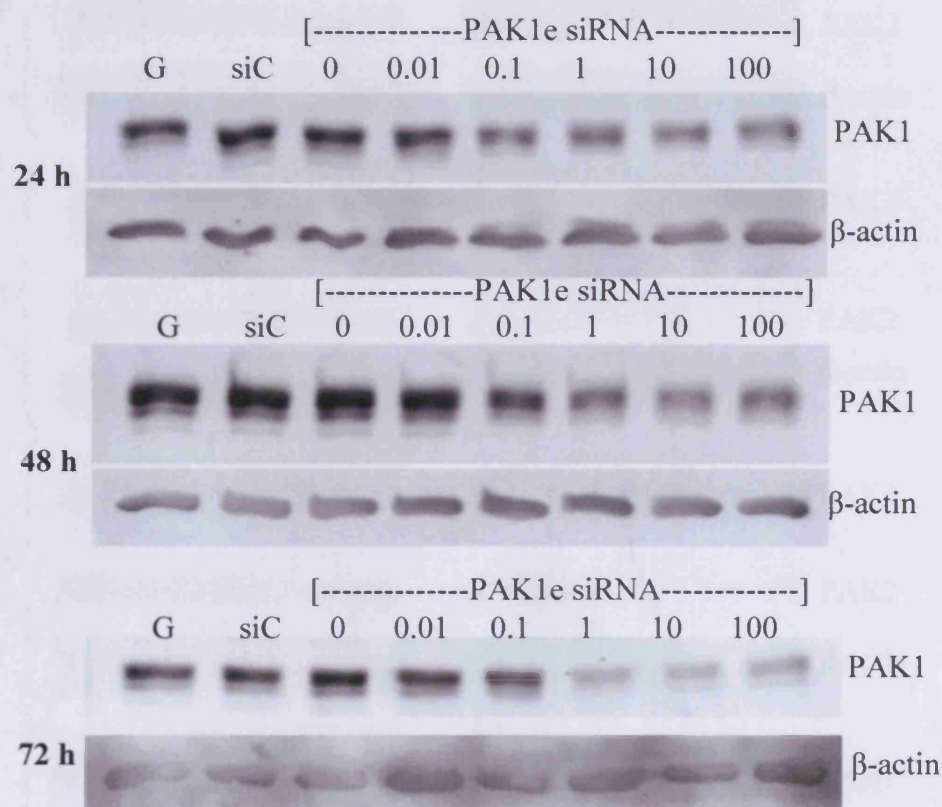


Figure 3.8: Titration of PAK1 siRNA in DU145 cells. DU145 cells were untransfected (G) or transfected with Control siRNA (siC) or siRNA against PAK1 (oligomer e) at concentrations from 0 to 100 pmoles/well in a 24-well plate. Lysates were prepared after 24, 48 or 72 hours and western blots were performed and probed with antibodies against PAK1 and β -actin.

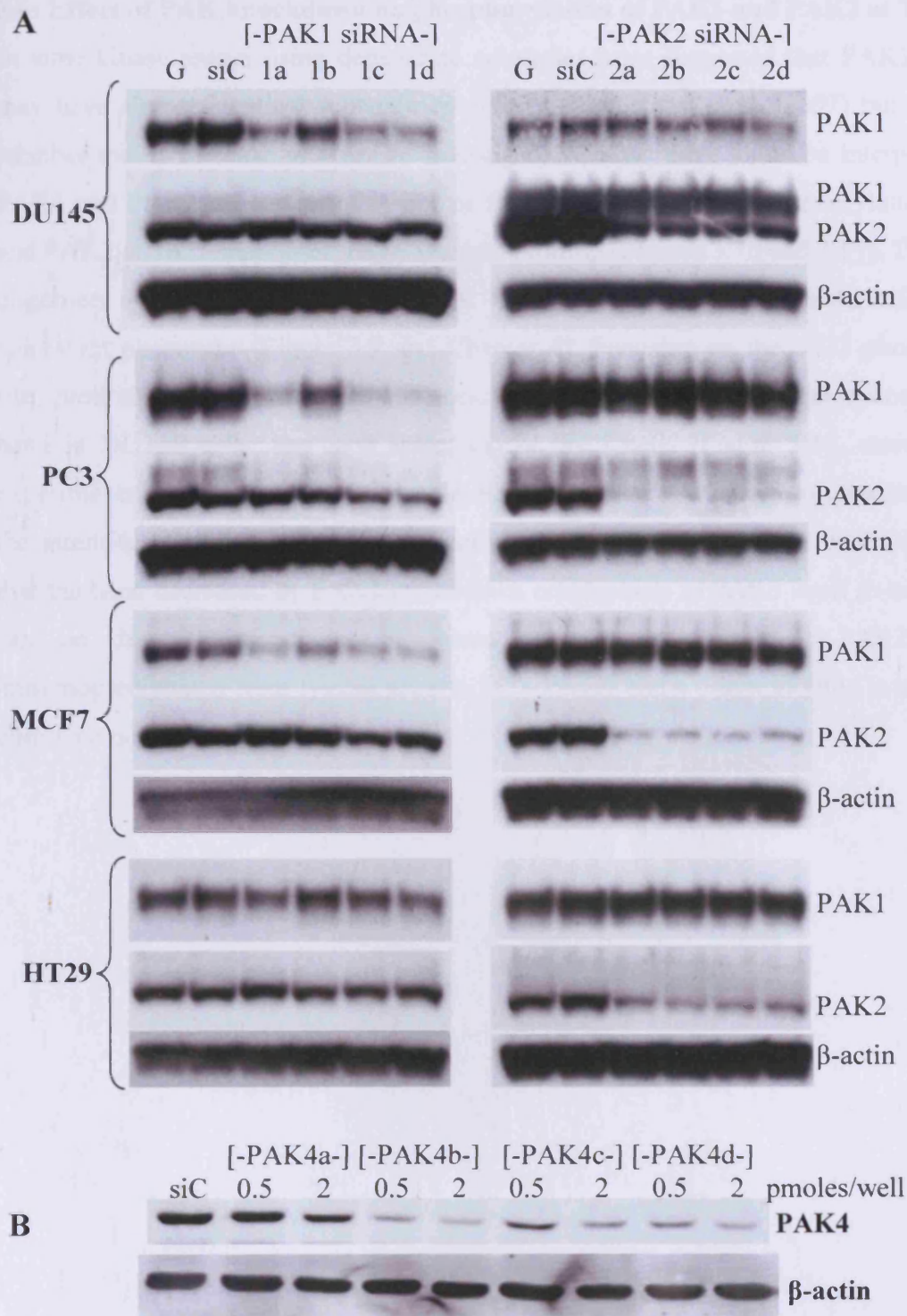


Figure 3.9: Knockdown of individual isoforms of PAK in cancer cells (A) Control siRNA (Quiagen) (siC) or one of four separate siRNAs against PAK1 or PAK2 were transfected into DU145, PC3, MCF7 or HT29 cells. Cell lysates were prepared after 48 hours and western blots were performed. Membranes were probed with antibodies against PAK1, PAK2 and β -actin. **(B)** Control siRNA (Quiagen) or one of four different siRNAs against PAK4 were transfected into DU145 cells. Lysates were prepared after 48 hours, western blots were performed and probed with antibodies against PAK4 and β -actin.

3.6: Effect of PAK knockdown on phosphorylation of PAK1 and PAK2 at T423

In vitro kinase assays using degenerate substrates have suggested that PAK1 and PAK2 may have almost identical substrate specificity (Rennefahrt et al., 2007) but it is unclear whether this is the case *in vivo*. To investigate whether there could be interplay between PAK1 and PAK2, the effect of PAK1 or PAK2 knockdown on phosphorylation of PAK1 and PAK2 at T423 was observed by western blotting (Figures 3.10 and 3.11). Two different oligomers were chosen for each PAK isoform based on knockdown efficiency and consistent phenotype (Figure 3.9 and Chapter 4). Focusing on the T423 phosphorylation site, preliminary data showed that knockdown of PAK2 decreased phosphorylation of a band in DU145 cells, and to a lesser extent, PC3 cells (Figure 3.11, asterisks). More experiments are required to confirm this. Knockdown of PAK1 had no consistent effect on the intensity of bands detected by the antibody (Figure 3.10). These results do not prove that the band decreased by PAK2 knockdown corresponds to PAK2 itself so no conclusion can be drawn from them. In future experiments, PAK1 or PAK2 could be immunoprecipitated from lysates prior to SDS-PAGE and western blotting in an attempt to eliminate non-specific bands.

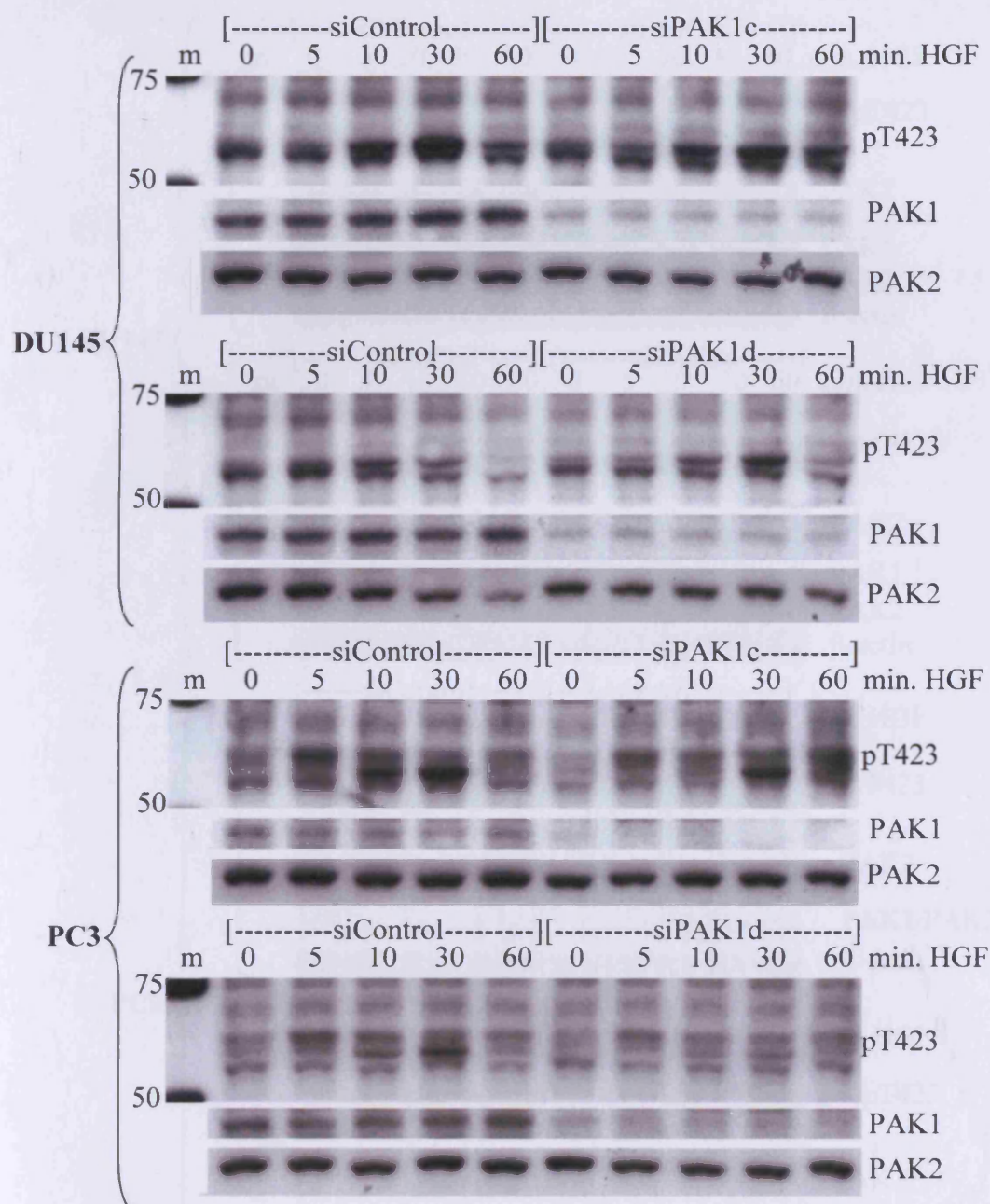


Figure 3.10: Effect of PAK1 knockdown on bands detected by the pT423 antibody (Abcam). PC3 or DU145 cells were treated with control siRNA or one of two separate single oligomers of siRNA against PAK1 prior to stimulation with 10 ng/ml HGF for 0 – 60 minutes. Lysates were prepared, western blots were performed and probed with an antibody against pT423 (Abcam), then reprobed with antibodies against PAK1 and PAK2. m=molecular weight markers (kDa). Some control blots are the same between Figures 3.10, 5.2, 5.17, 5.23 and 5.21. Data are from one experiment for pT423.

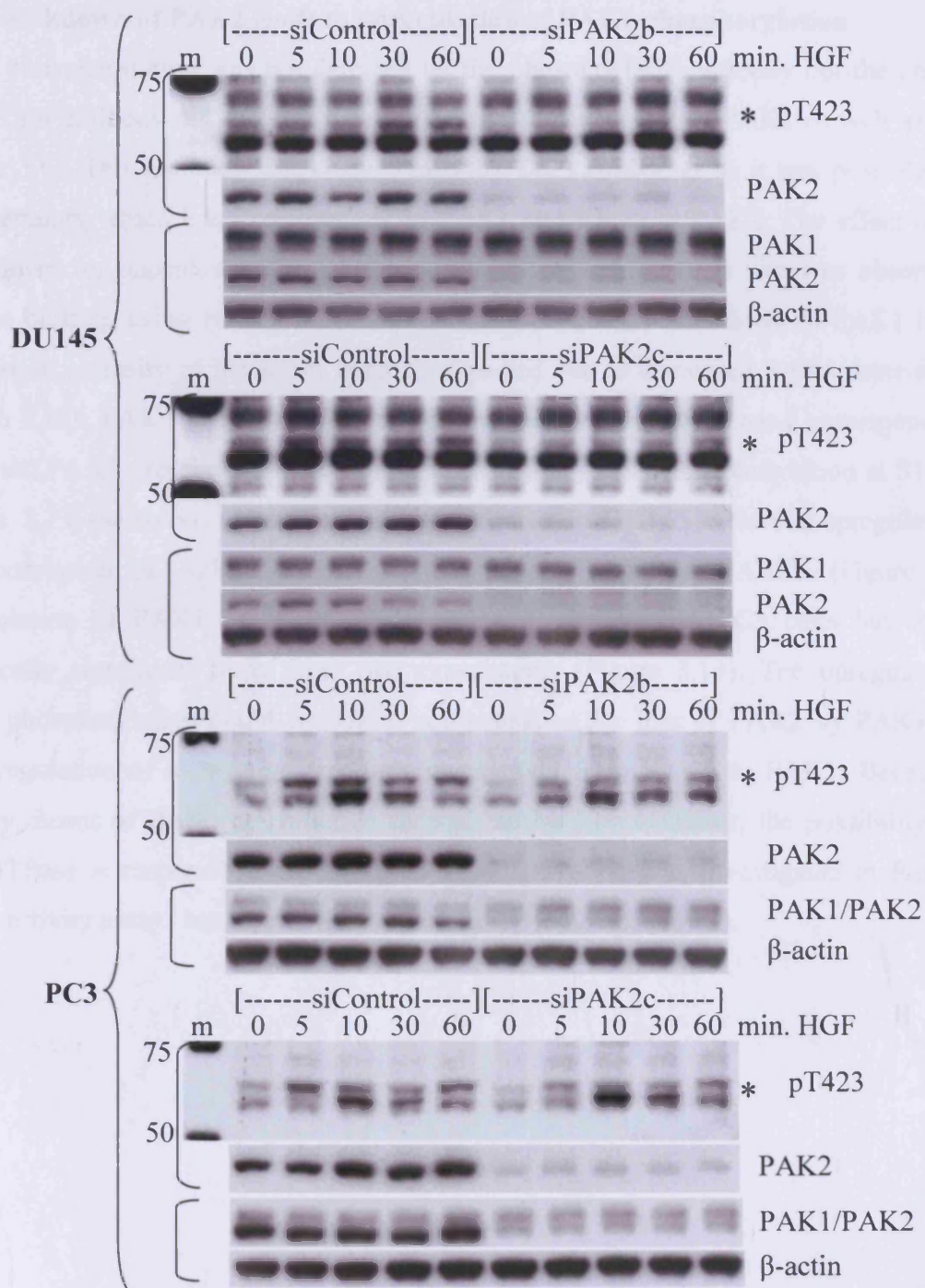


Figure 3.11: Effect of PAK2 knockdown on bands detected by the pT423 antibody (Abcam). PC3 or DU145 cells were treated with control siRNA or one of two separate single oligomers of siRNA against PAK2 prior to stimulation with 10 ng/ml HGF for 0 – 60 minutes. Lysates were prepared, western blots were performed and probed with antibodies against pT423 (Abcam), and PAK2 or PAK1, PAK2 and β-actin as knockdown controls. m=molecular weight markers (kDa). Straight left brackets indicate reprobes of the same membrane. Some control bands are the same between Figures 3.11 3.13, 5.9, 5.18, 5.22 and 5.24. Data are from one experiment for pT423.

3.7: Knockdown of PAK2 leads to upregulation of PAK1 phosphorylation

PAK1 phosphorylation was not detected by the phospho-T423 antibody but the phospho-S199/S204 antibody did detect a band at the molecular weight of PAK1 as well as PAK2 (Figure 3.6). This antibody was cleaner than the T423 antibodies so it was possible to say with certainty which band corresponds to PAK1 and which to PAK2. The effect of PAK knockdown on phosphorylation of the S199/204 phosphorylation site was observed by western blotting using lysates from DU145 and PC3 cells. Knockdown of PAK1 led to a decrease in intensity of the upper band as expected due to decreased PAK1 protein levels (Figure 3.12). PAK2 knockdown decreased intensity of the lower band corresponding to decreased PAK2 protein expression but also increased PAK1 phosphorylation at S199/204 (Figure 3.13 asterisks). Quantification showed that in DU145 cells the upregulation of phosphorylation at S199/204 was significant for oligomer PAK2b (Figure 3.14). Upregulation of PAK1 phosphorylation was also observed in PC3 cells but was not statistically significant from these two experiments (Figure 3.14). The upregulation of PAK1 phosphorylation could be due to compensation for loss of PAK2 by PAK1 or by down-regulation of a negative feedback mechanism from PAK2 to PAK1. Because the primary means of PAK1 activation is through active Rho GTPases, the possibility that a Rho GTPase is responsible for increased PAK activation was investigated in Rac1 and Cdc42 activity assays but results were inconclusive (data not shown).

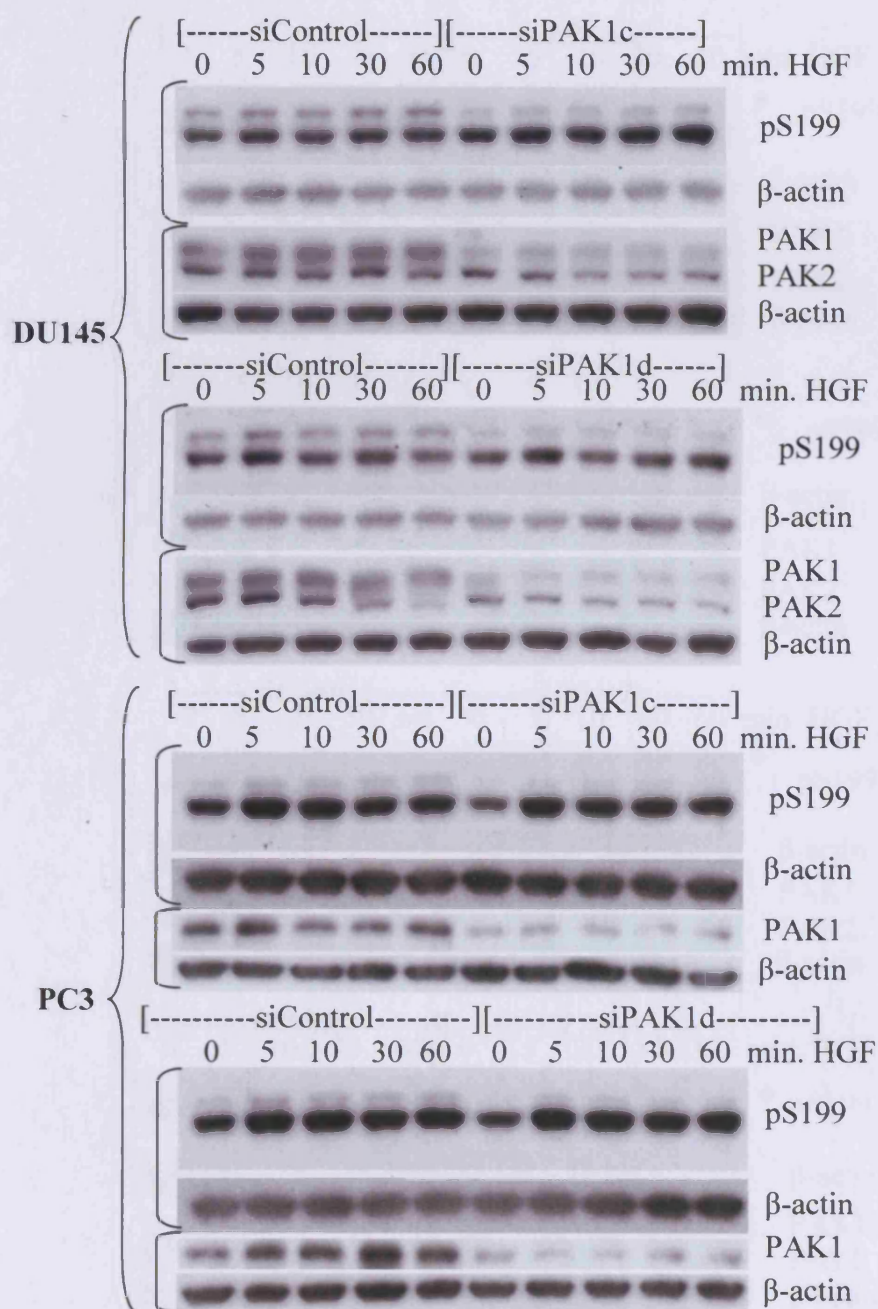


Figure 3.12: PAK1 knockdown does not affect PAK2 phosphorylation at S199/204. Western blots were performed using lysates prepared for Figure 3.10 and probed with antibodies against pS199/204 and β -actin or PAK1, PAK2 and β -actin (DU145) or PAK1 and β actin (PC3). Straight left brackets indicate reprobes of the same membrane. Some control blots are the same between Figures 3.12, 5.17 and 5.23. Blots are representative of two independent experiments

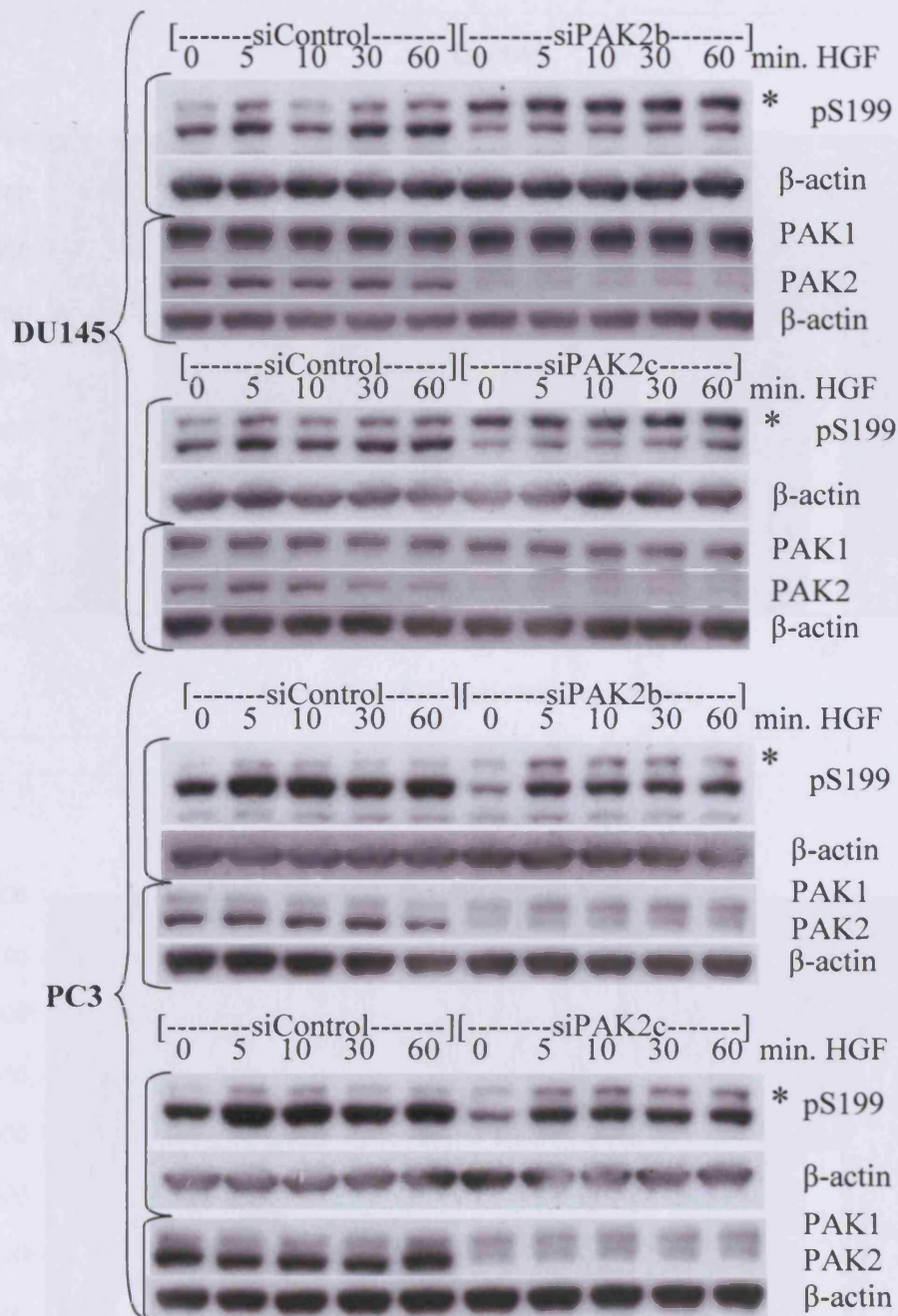


Figure 3.13: PAK2 knockdown leads to increased PAK1 phosphorylation at S199/204. Western blots were performed using lysates prepared for Figure 3.11, and probed with antibodies against pS199/204 and β-actin, or PAK1, PAK1, PAK2 and β-actin as knockdown controls. Note the knockdown control blots are the same as in Figure 3.11. Asterisks indicate the band corresponding to pPAK1 (S199/204). Straight left brackets indicate reprobes of the same membrane. Some control bands are the same between Figures 3.11 3.13, 5.9, 5.18, 5.22 and 5.24. Blots are representative of two independent experiments.

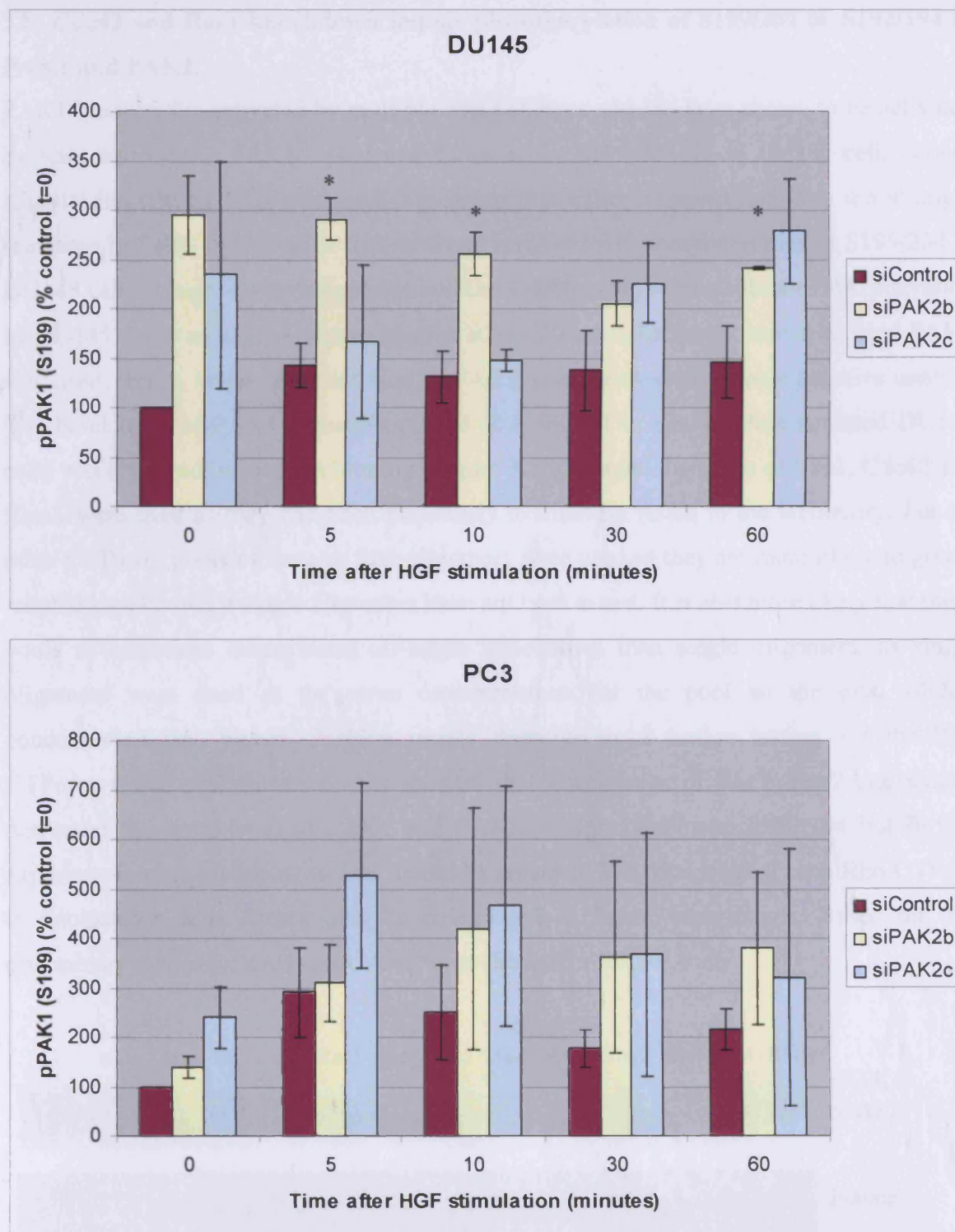


Figure 3.14: Quantification of the effect of PAK2 knockdown on PAK1 phosphorylation at S199/204. Blots were quantified using ImageJ software. PAK1 and pPAK1 (S199) bands were first normalised to actin loading controls, then pPAK1 was normalised to PAK1 level. Data are from two independent experiments, error bars represent the two means from each condition. Control results were pooled and represent four means from two independent experiments. * = $p < 0.05$, two tailed student's T-test.

3.8: Cdc42 and Rac1 knockdown impair phosphorylation of S199/204 or S192/194 in PAK1 and PAK2.

PAK1 binds and is activated by multiple Rho GTPases and has been shown to be activated by Rac1 and Cdc42. PAK1/2 phosphorylation at the S199/204 site in DU145 cells is only slightly elevated by HGF stimulation meaning that either phosphorylation is not strongly regulated by HGF signalling or that the basal level of PAK phosphorylation at S199/204 in DU145 cells is high. To investigate which Rho GTPases are responsible for PAK activation in DU145 cells, an siRNA library against Rho GTPases previously shown to bind PAK1 was used. RhoA, which does not bind to PAKs, was knocked down as a negative control. The basal level of PAK1/2 phosphorylated at S199/204 in Rho-GTPase depleted DU145 cells was observed by western blotting (Figure 3.15). Single oligomers of Rac1, Cdc42 and RhoA were used as they had been previously extensively tested in the laboratory. For all other GTPases, pools of three or four oligomers were used as they are more likely to give a reliable knockdown if single oligomers have not been tested. It is also more likely that these pools of oligomers cause more off-target knockdown than single oligomers, as single oligomers were used at the same concentrations for the pool so the total siRNA concentration was higher. Positive results therefore need further testing. Of the Rho GTPases tested preliminary results showed that knockdown of Rac1, Rac2 and Cdc42 decreased the basal level of PAK1 and PAK2 phosphorylation at S199/204 but further experiments were inconclusive and should be repeated. The total level of each Rho GTPase in knockdown cells should also be determined in future experiments. Based on this preliminary evidence, Rac1 and Cdc42 were chosen for further study.

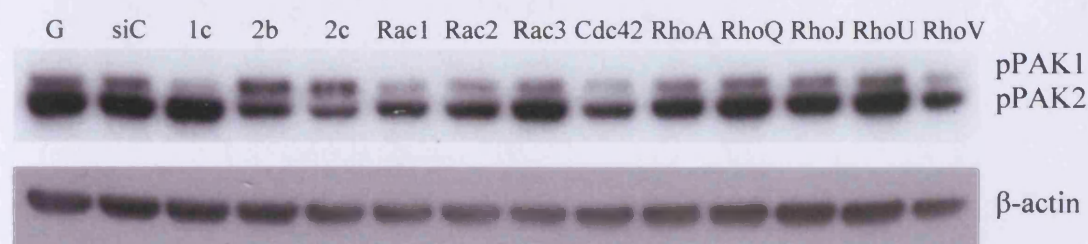


Figure 3.15: Cdc42 and Rac1 affect activity of PAK1 and PAK2: DU145 cells were treated with control siRNA, single oligomers of PAK1 (1c), PAK2 (2b and 2c), Rac1, Cdc42 or RhoA, or pools of three or four oligomers of RhoJ, RhoQ, RhoU or RhoV siRNA. After 48 hours lysates were prepared, western blots were performed and probed with antibodies against PAK1/PAK2 phosphorylated at S199/204 and actin.

3.9: Cdc42 and Rac1 impair PAK phosphorylation downstream of HGF

In order to determine the importance of Rho GTPases in activating PAK1 and PAK2 downstream of HGF, siRNA was used to knock down Rac1 or Cdc42 in DU145 or PC3 cells, and PAK1 or PAK2 phosphorylation was observed following HGF stimulation by western blotting. Firstly, phosphorylation was observed using the phospho-T423 antibody. In DU145 cells, preliminary results were inconclusive; knockdown of Rac1 (Figure 3.16) or Cdc42 (Figure 3.17) slightly decreased the intensity of the band close to the molecular weight of PAK2 but further evidence is required to show whether this corresponds to PAK2. In PC3 cells, the effect of Rac1 or Cdc42 was unclear due to low intensity of the relevant band. Further experiments could be performed to provide evidence for the effect of Rac1 or Cdc42-depletion on PAK1/2 phosphorylation at T423. For example, as previously mentioned, PAK1 or PAK2 could be immunoprecipitated from lysates prior to SDS-PAGE and western blotting to limit non-specific binding of the antibody. A slight reduction in the total level of PAK2 was also seen in Cdc42-depleted DU145 cells but this was not observed in other experiments using the same oligomers.

It should also be noted that Rac1 and Cdc42 ran as doublets on SDS-PAGE gels under these conditions. The reason for this was not clear and has not been previously investigated. It could depend on the lysis buffer or sample buffer composition.

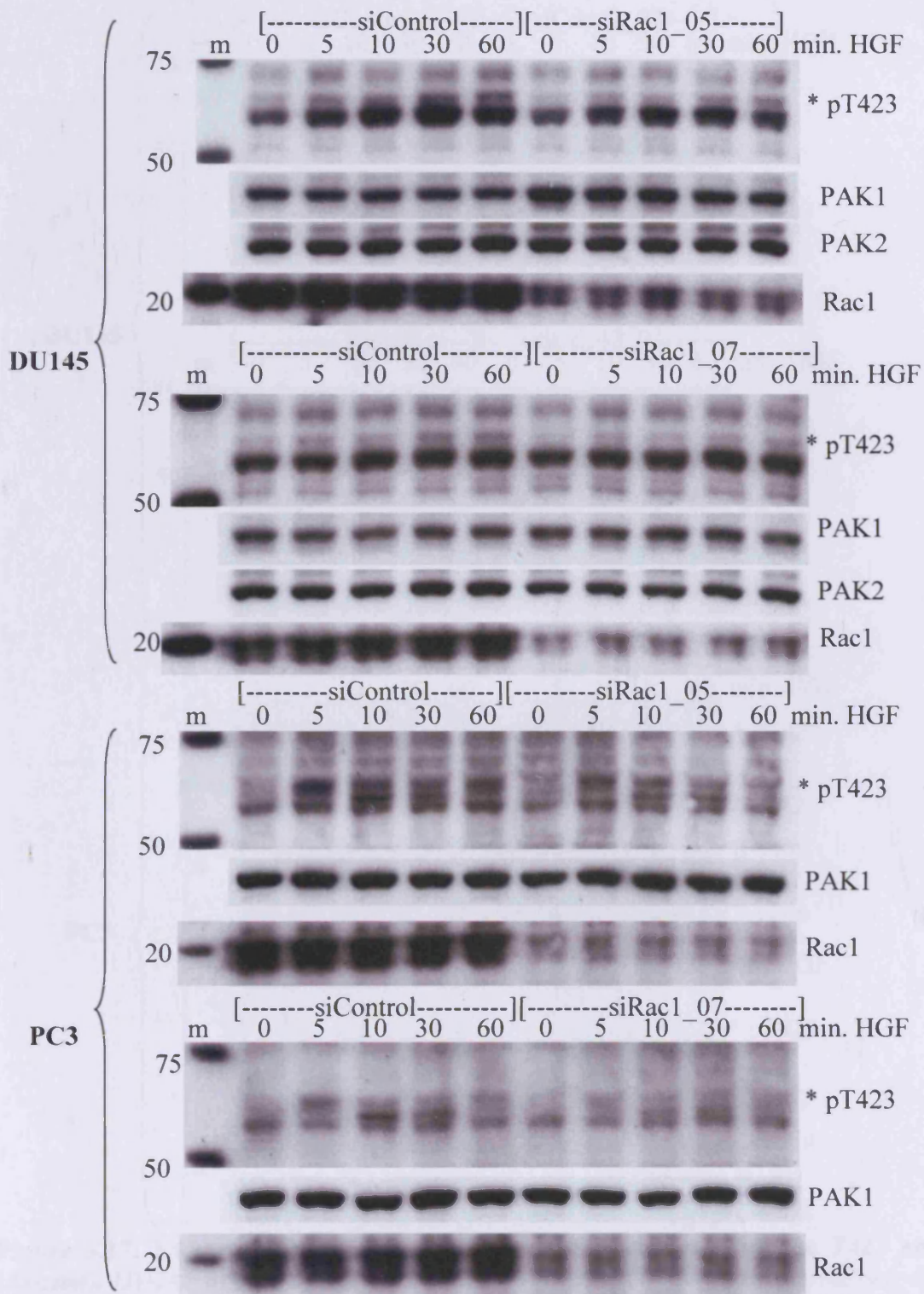


Figure 3.16: Effect of Rac1 knockdown on bands detected by the T423 antibody (Abcam). DU145 or PC3 cells were transfected with control siRNA or one of two siRNA oligomers against Rac1. After 48 hours, cells were treated with 10 ng/ml HGF for 0 – 60 minutes and lysates were prepared. Western blots were performed and probed with antibodies against pT423, PAK1, Rac1 (PC3) or pT423, PAK1, PAK2 and Rac1 (DU145). Data are from one experiment.

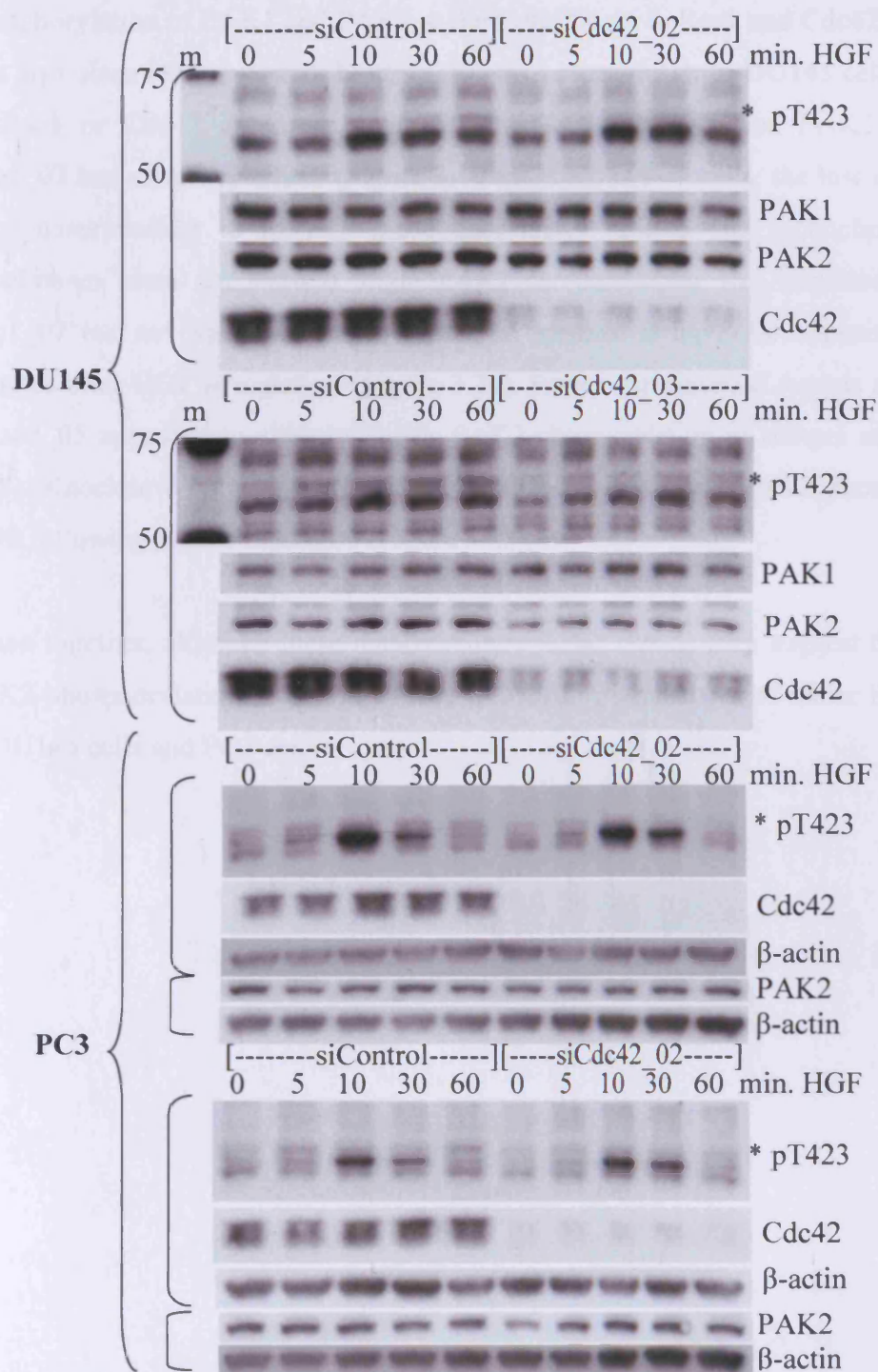


Figure 3.17: Effect of Cdc42 knockdown on bands detected by the T423 antibody (Abcam). DU145 or PC3 cells were treated with control siRNA or one of two different oligomers of siRNA against Cdc42. After 48 hours, cells were treated with 10 ng/ml HGF for 0 – 60 minutes and lysates were prepared. Western blots were performed and probed with antibodies against pT423, PAK1, PAK2 and Rac1 (DU145), or pT423, Cdc42 and β -actin, or pT423, Cdc42 and β -actin (PC3). Asterisks mark the position of the minor band affected by Rac1 or Cdc42 knockdown. Straight left brackets indicate re-probes of the same membrane. Data are from one experiment

Phosphorylation of PAK1 and PAK2 at the S199/204 site in Rac1 and Cdc42-depleted cells was also observed by western blotting (Figure 3.18 and 3.21). In DU145 cells, knockdown of Rac1 or Cdc42 impaired phosphorylation of both PAK1 and PAK2 but oligomer Rac1_07 had a weaker effect. (Figure 3.19 and 3.22). In PC3 cells, the low intensity of the band corresponding to pPAK1 made data highly variable and inconclusive for Rac1 knockdown (data not shown) while PAK2 phosphorylation was impaired by oligomer Rac1_07 but not Rac1_05. In fact Rac1_05 appeared to have the opposite effect at 10 minutes after HGF stimulation (Figure 3.20). Further experimental repeats are required as siRac1_05 appeared to slightly impair PAK2 phosphorylation in images of blots (Figure 3.18). Knockdown of Cdc42 in PC3 cells impaired PAK1 and PAK2 phosphorylation at S199 following HGF stimulation (Figure 3.23).

Taken together, although these results require further repeats they suggest that PAK1 and PAK2 phosphorylation at S199/204, are impaired by knockdown of either Rac1 or Cdc42 in DU145 cells and PC3 cells.

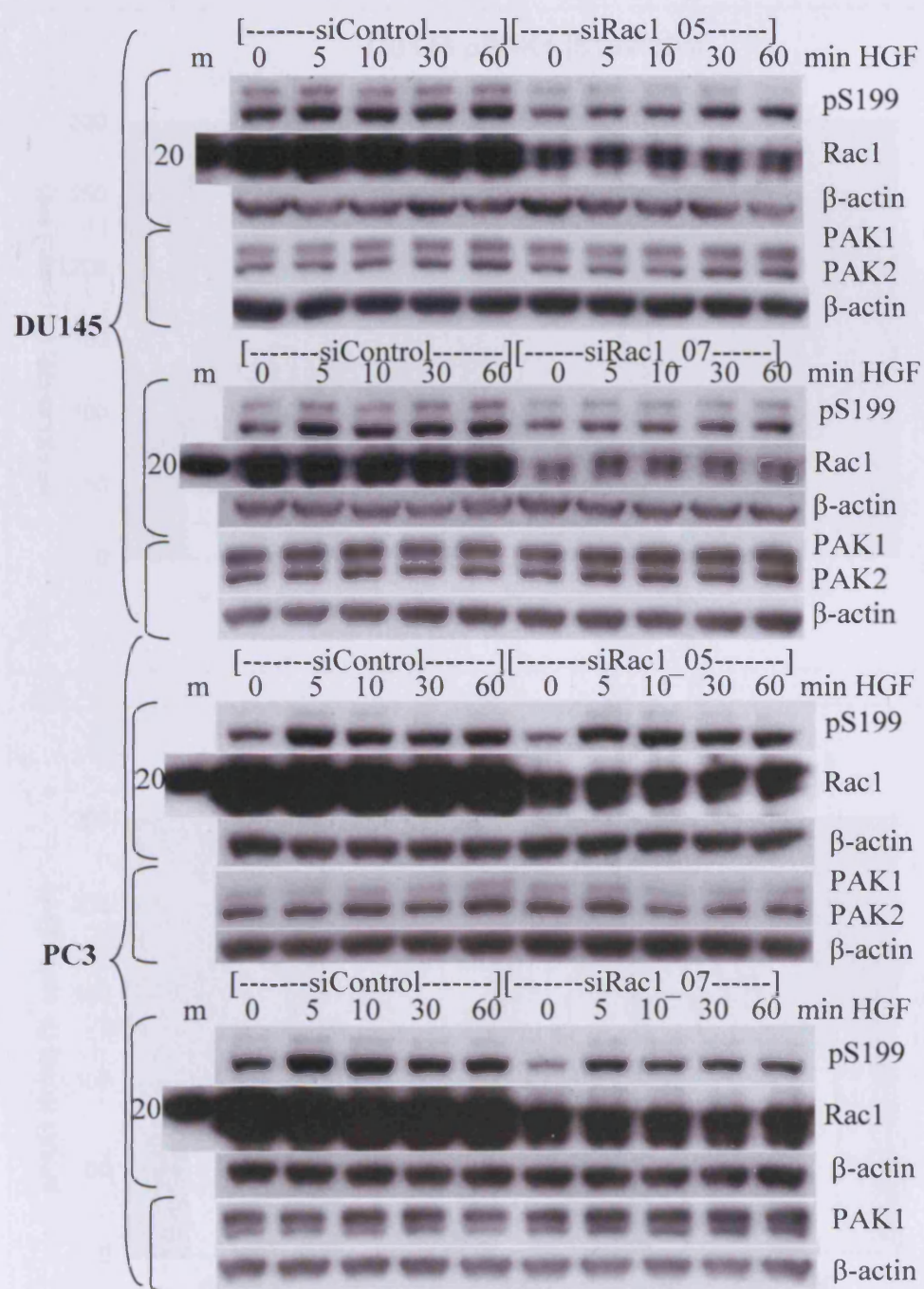


Figure 3.18: Knockdown of Rac1 reduces phosphorylation of PAK1 and PAK2 at S199/204 downstream of HGF. Western blots were performed using lysates prepared for Figure 3.16 and probed with antibodies against pS199/204, Rac1, and β-actin, or PAK1, PAK2 and β-actin, or PAK1 and β-actin (PC3, siRac1_07). Blots are representative of two independent experiments. Straight left brackets indicate reprobes of the same membrane.

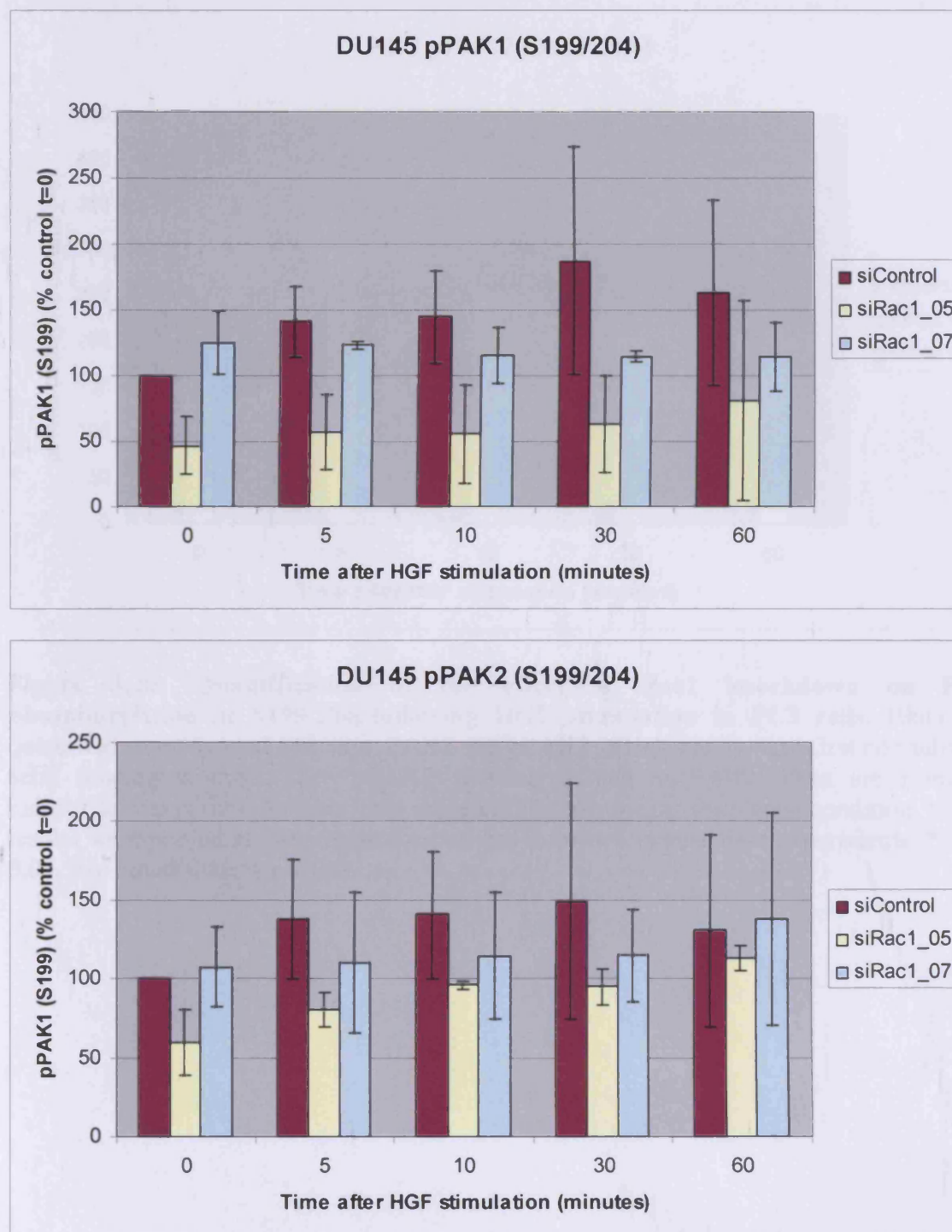


Figure 3.19: Quantification of the effect of Rac1 knockdown on PAK1 or PAK2 phosphorylation at S199/204 following HGF stimulation in DU145 cells. Blots were quantified using ImageJ software. PAK1 and pPAK1, or PAK2 and pPAK2 (S199) bands were first normalised to actin loading controls, then pPAK1 was normalised to the PAK1 and pPAK2 was normalised to PAK2. Data are from two independent experiments, error bars represent the two means from each condition. Control results were pooled and represent four means from two independent experiments. T-tests did not reveal statistical significance

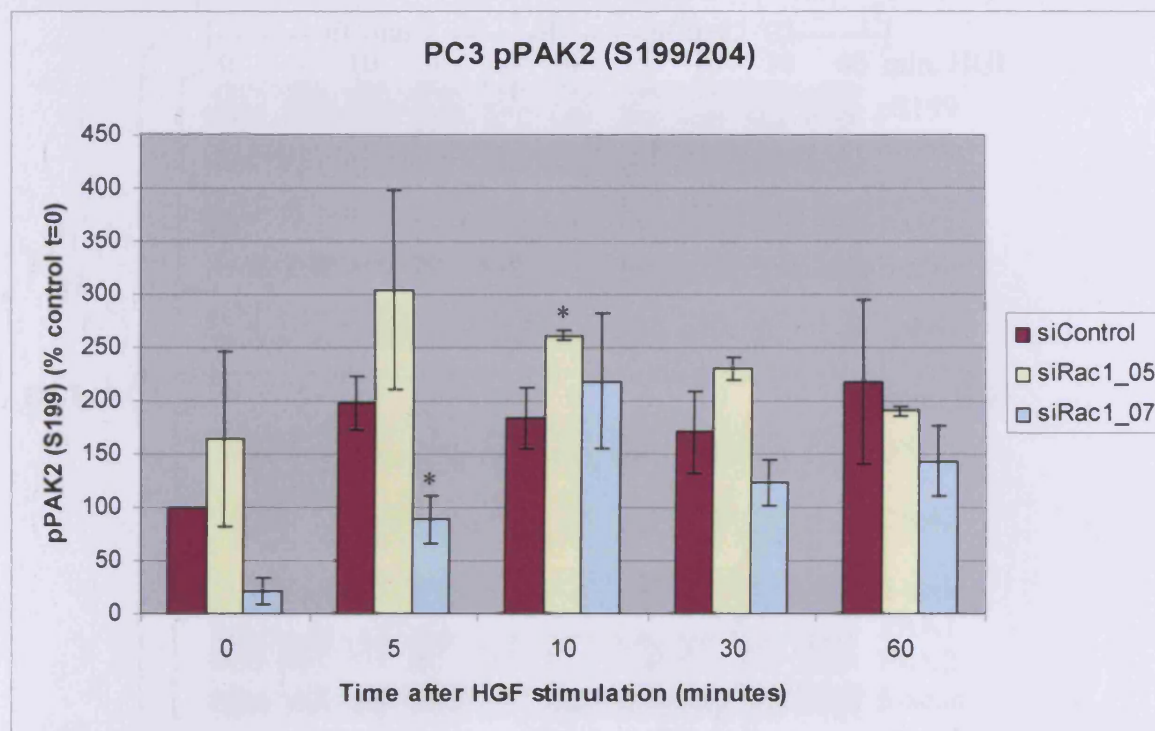


Figure 3.20: Quantification of the effect of Rac1 knockdown on PAK2 phosphorylation at S199/204 following HGF stimulation in PC3 cells. Blots were quantified using ImageJ software. PAK2 and pPAK2 (S199) bands were first normalised to actin loading controls, then pPAK2 was normalised to PAK2. Data are from two independent experiments, error bars represent the two means from each condition. Control results were pooled and represent four means from two independent experiments. * = $p < 0.05$, two tailed student's T test.

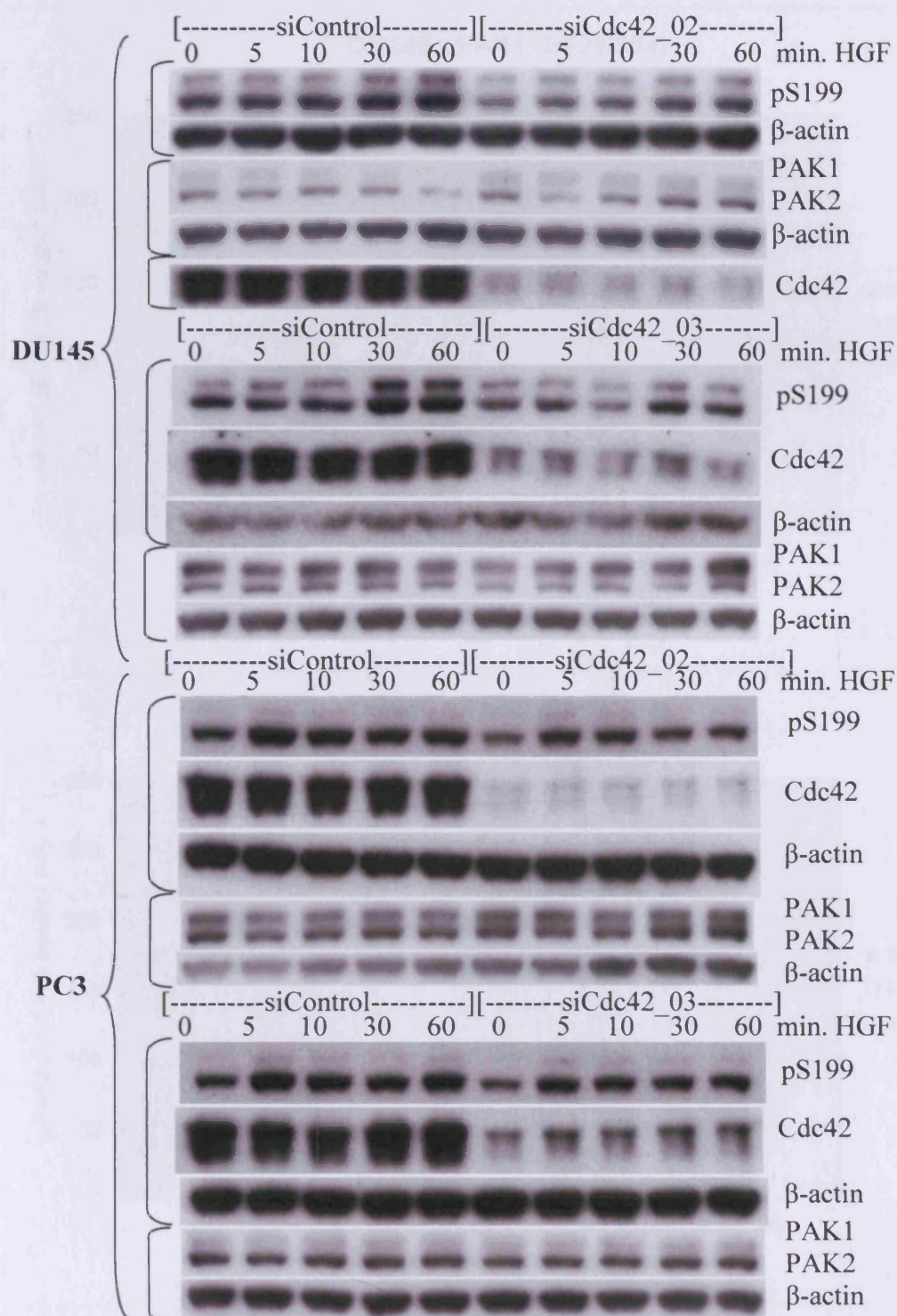


Figure 3.21: Knockdown of Cdc42 impairs phosphorylation of PAK1 and PAK2 at S199/204 downstream of HGF. Western blots were performed using lysates prepared for Figure 3.17 and probed with antibodies against pS199/204, Cdc42, β -actin PAK1 or PAK2. Straight left brackets indicate blots from the same membrane. Blots are representative of two independent experiments.

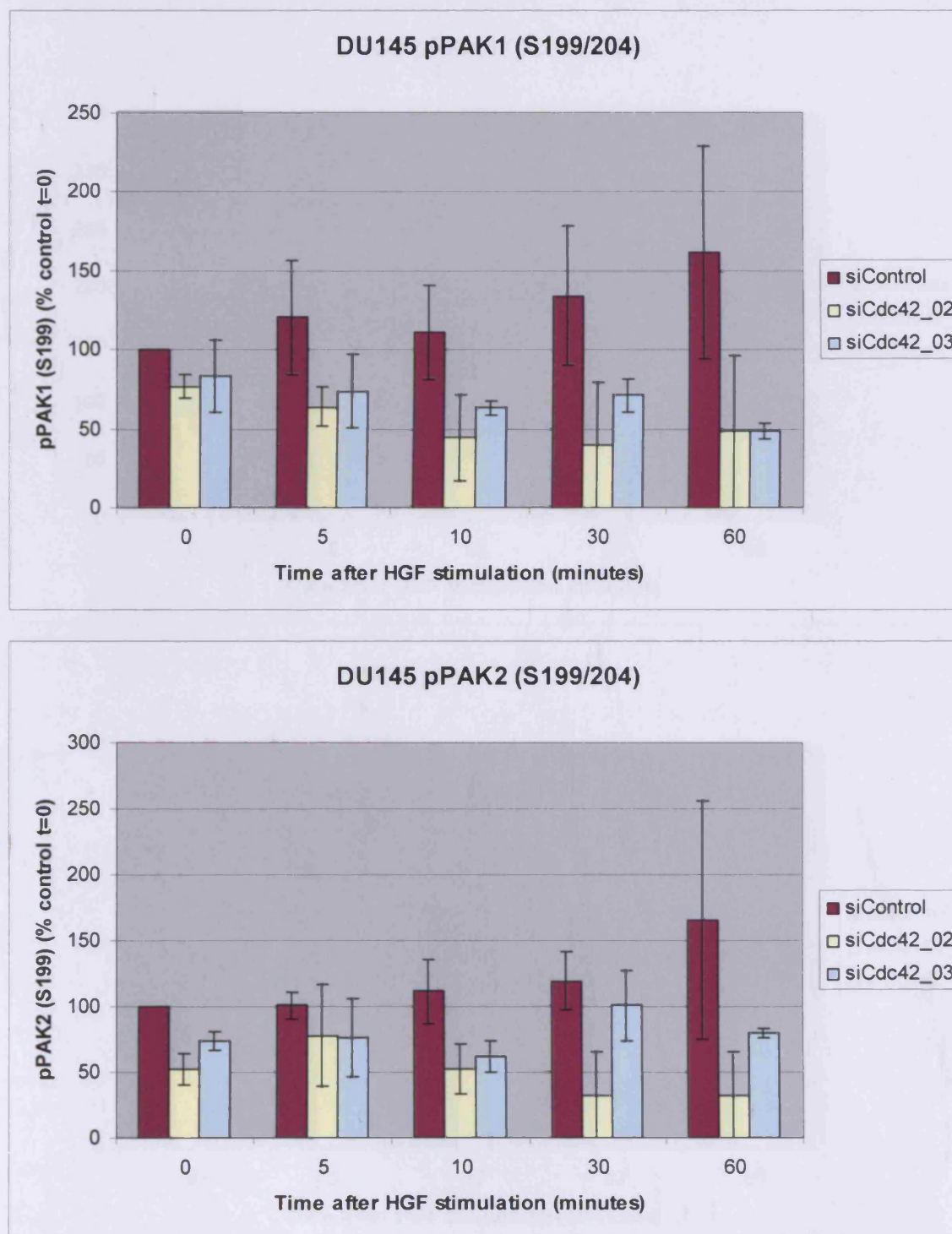


Figure 3.22: Quantification of the effect of Cdc42 knockdown on PAK1 or PAK2 phosphorylation at S199/204 following HGF stimulation in DU145 cells. Blots were quantified using ImageJ software. PAK1 and pPAK1, or PAK2 and pPAK2 (S199) bands were first normalised to actin loading controls, then pPAK1 was normalised to the PAK1 and pPAK2 was normalised to PAK2. Data are from two independent experiments, error bars represent the two means from each condition. Control results were pooled and represent four means from two independent experiments. T-tests did not reveal statistical significance

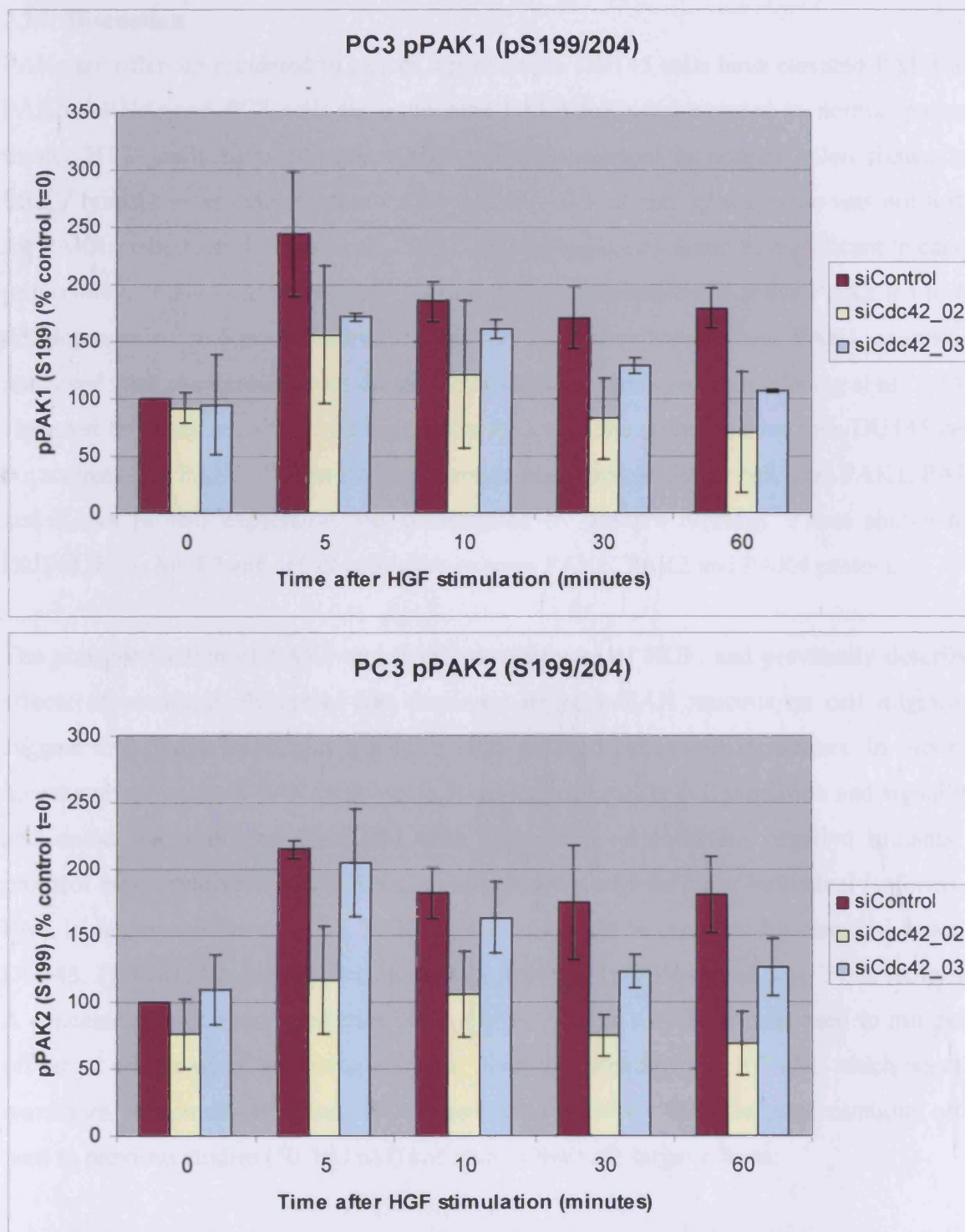


Figure 3.23: Quantification of the effect of Cdc42 knockdown on PAK1 or PAK2 phosphorylation at S199/204 following HGF stimulation in PC3 cells. Blots were quantified using ImageJ software. PAK1 and pPAK1, or PAK2 and pPAK2 (S199) bands were first normalised to actin loading controls, then pPAK1 was normalised to the PAK1 and pPAK2 was normalised to PAK2. Data are from two independent experiments, error bars represent the two means from each condition. Control results were pooled and represent four means from two independent experiments. T-tests did not reveal statistical significance

3.10: Discussion

PAKs are often up regulated in cancer, for example DU145 cells have elevated PAK4 and PAK6 mRNA, and PC3 cells have elevated PAK4 mRNA compared to normal prostate tissue; HT29 cells have elevated PAK4 mRNA compared to normal colon tissue; and MCF7 breast cancer cells express PAK4 mRNA – but normal breast tissue was not tested for PAK4 expression (Callow et al., 2002). This up regulation could be significant to cancer progression; *Wang et al.* previously showed that overexpression of active PAK1 led to the development of malignant mammary tumours in mouse models and PAK1 expression increased with progression from ductal hyperplasia to adenocarcinoma (Wang et al., 2006). However this may not always be the case as PC3 cells are more invasive than DU145 cells but express less PAK1. To test PAK expression in a panel of cancer cell lines PAK1, PAK2 and PAK4 protein expression was investigated by western blotting. It was shown that DU145, PC3, MCF7 and HT29 cells each express PAK1, PAK2 and PAK4 protein.

The phosphorylation of PAK1 and PAK2 downstream of HGF, and previously described effects of constitutively active and dominant negative PAK mutants on cell migration, suggest that PAKs could play a role in HGF-induced cancer cell responses. In order to investigate the roles of PAK isoforms in HGF-induced cancer cell migration and signalling and avoid the problems associated with expression of dominant negative mutants, a protocol was established to use RNAi to knock down expression of individual isoforms of PAK in cancer cell lines. PAK1, PAK2 and PAK4 could be reproducibly knocked down in DU145, PC3, MCF7 and HT29 cells without affecting expression of other PAK isoforms. A concentration of short interfering RNA (siRNA) equal to 3.3 nM was used to minimise off-target effects while achieving a similar level of knockdown to 167 nM, which was the maximum concentration tested. This is considerably lower than the concentrations often used in previous studies (50-100 nM) and should limit off-target effects.

In MDCK cells, HGF stimulates scattering and activates overexpressed PAK1 and PAK4 (Royal et al., 2000; Wells et al., 2002). DU145 cells have also been shown to scatter in response to HGF but PAK activity has not been studied so far (Miura, 2001). Thus PAKs could be potential therapeutic targets if they also control HGF-induced invasion *in vivo*. Activation of group I PAKs begins with dissociation of the autoinhibited dimer followed by phosphorylation at multiple sites which increase kinase activity, lock the kinase in an active

state or modulate adapter binding (See chapter 1, Figure 1.9). Phosphorylation of the activation loop of group I PAKs is required for full activation (Threonine 423 in PAK1) (Zenke et al., 1999). Two different antibodies described to recognise PAK1, PAK2 and PAK3 phosphorylated at T423 were tested (CST and Abcam) and each detected multiple bands over a wide range of molecular weights. PAKs are part of a larger family of Ste20-related kinases (STKs) and it is likely that many of the bands detected using pT423 antibodies are other Ste20 kinases. The antibody from CST has been described to cross-react with Mst1 (STK4) and Mst2 (STK3) phosphorylated at T183 or T180 respectively. These sites share only 46% identity with the peptide used to raise the antibody (EQSKRST_pMVGTPY) but using BLAST with the BLOSUM62 scoring matrix gives 85% positive scores showing that many of the unmatched amino acids have similar characteristics (Figure 3.24A). It is likely that both pT423 antibodies will also recognise other STKs with homology in this region. BLAST searching using the PAK1 amino acid sequence as a template and looking for similarity at the T423 region reveals that there are a number of possible candidates that could cross react with the antibody. Notably STK24, STK25 and Ste20-like kinase (SLK) have over 60% positive scores in the BLAST search. Unrelated proteins with homology in this region were also identified, including Myosin IIIa, MAP4K3 and MAP4K5 (Figure 3.24B).

A	PAK1/2/3 peptide	EQSKRSTpMVGTPY
		+KR+Tp++GTP+
	STK3/STK4	TMAKRNTpVIGTPF
<hr/>		
B	PAK1/2/3	EQSKRSTMVGTPY
		Q KR+T VGTP+
	STK24/25	TQIKRNTFVGTPF
	PAK1/2/3	EQSKRSTMVGTPY
		+R + +GTPY
	SLK	TIQRRDSFIGTPY
	PAK1/2/3	EQSKRSTMVGTPY
		+ +R+T VGTP+
	Myosin IIIa	TRHRRNTSVGTPF
	PAK1/2/3	EQSKRSTMVGTPY
		+KR + +GTPy
	MAP4K3/5	TIAKRRSFIGTPY

Figure 3.24: STK3/4 homology with PAK1/2/3 peptide used to generate pT423 antibodies. A) Sequence alignment of the PAK1/2/3 phosphopeptide surrounding T423 and the STK3/STK4 region surrounding T180/T183. B) Sequence alignment of the T423 region of PAK1 and other homologous proteins which could be recognised by the pT423 antibody. + represents a positive score in the BLAST search using the BLOSUM62 scoring matrix.

Lot 3 of the CST pT423 antibody detected phosphorylation of a protein running close to PAK1 on western blots. It is possible that this corresponded to phospho-PAK1 although

this was not validated by the use of siRNA and a different batch of the antibody did not detect the band. The Abcam pT423 antibody did not detect this band either, showing that differences in preparation and batch lead to differences in the affinity of the final antibody for the antigen. However, the Abcam pT423 antibody did detect phosphorylation of protein migrating at the molecular weight of PAK2 which decreased with PAK2 knockdown in a preliminary experiment. The absence of a band corresponding to pPAK2 when using the CST antibody and pPAK1 when using the Abcam antibody suggests that there may be a slight structural difference between PAK1 and PAK2 in this region even though the sequence is identical.

As an alternative to the pT423 antibody, an antibody described to detect PAK1 and PAK2 phosphorylated at S199/204 or S192/197 respectively was used. It is thought that this is an autophosphorylation site but it cannot be ruled out that it is also phosphorylated by another kinase or another PAK isoform although this has not been shown. This antibody was cleaner than the pT423 antibodies and the identity of the bands was validated using siRNA against PAK1 or PAK2. Western blots showed that phosphorylation of PAK1 and PAK2 at S199/204 increased with HGF stimulation and decreased following knockdown of the corresponding PAK isoform. Taken together, the data from both antibodies show that PAK1 and PAK2 are phosphorylated and probably activated downstream of HGF in the cell lines tested.

RNAi was used to investigate interplay between PAK1 and PAK2 in DU145 and PC3 cells. Knockdown of PAK1 had no effect on phosphorylation of PAK2 at S192/197 observed using a pS199/204 antibody respectively. However, in both DU145 and PC3 cells PAK2 knockdown led to an increase in PAK1 phosphorylation at S199/204. This suggests that PAK2 negatively regulates PAK1 phosphorylation at this site, which has not been previously described. S199/204 are autophosphorylation sites but could also be substrates for other kinases. Therefore PAK2 could either negatively regulate the phosphorylation of PAK1 directly or the activity of an upstream kinase or phosphatase with S199/204 as a substrate (Figure 3.25). To investigate whether PAK2 knockdown could lead to activation of PAK1 through Rac1 or Cdc42, pull down assays to assess Cdc42 and Rac1 activity were performed but were inconclusive and should be repeated in future work. Another possibility for the mechanism of PAK1 regulation by PAK2 could be if PAK2 can dimerise with

PAK1 and block phosphorylation. Whether this is the case is unclear, studies of PAK2 dimerisation showed that residues in the kinase domain of PAK2 that interact with the autoinhibitory domain are identical to those in the kinase domain of PAK1 (Pirrucello et al., 2006). However, the dimerisation domain in the regulatory region of PAK1 shares only 30% identity with the region in PAK2 (Bokoch 2003) and PAK1 knockdown did not affect PAK2 phosphorylation at S199/204. A possible future direction for experiments could be to directly investigate whether PAK2 and PAK1 can heterodimerise.

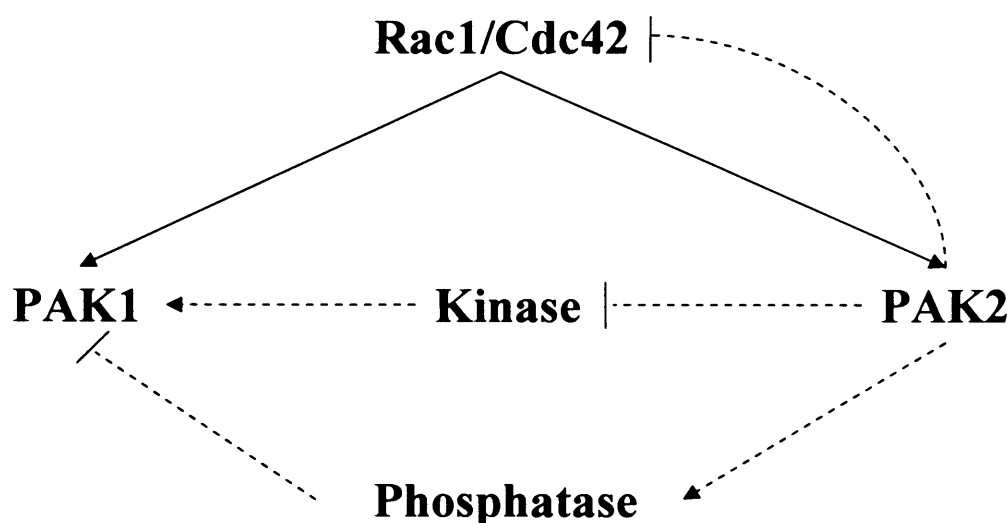


Figure 3.25: Hypothetical pathways for the regulation of pS199/204 on PAK1 by PAK2. Knockdown of PAK2 upregulates phosphorylation of PAK1 at S199/204 by an unknown mechanism suggesting that PAK2 downregulates PAK1. This could be through negative feedback from PAK2 to Rac1/Cdc42 leading to decreased activation and autophosphorylation of PAK1; or through PAK2-mediated inhibition of a kinase or activation of a phosphatase with pS199/204 as a substrate.

Phosphorylation of PAK4 at S474 was not observed following HGF stimulation but PAK4 activation was expected as it has been shown to be activated downstream of Cdc42 in HGF-stimulated MDCK cells. Phosphorylation of PAK4 at S474 could therefore be cell type specific, but also the physiological relevance of Group II PAK phosphorylation at S474 is unclear and it may not be a readout of kinase activity. *In vitro* kinase assays on immunoprecipitated PAKs would be the best way to study group II PAK activation following HGF stimulation (Wells 2002).

To investigate upstream activators of PAK1/2 which could be responsible for their activation downstream of HGF, siRNA was used to knock down Rho GTPases previously shown to bind to PAK1 or PAK2 (see Chapter 1, Table 2). Of the Rho GTPases tested in a preliminary experiment, Rac1, Rac2 and Cdc42 decreased the basal level PAK1 and PAK2 phosphorylated at S199/204 in DU145 cells while Rac3, RhoA, RhoJ (TCL), RhoQ (TC10), RhoU (Wrch-1) and RhoV (Chp) had no effect. In DU145 cells, so far only Rac1, Rac3, RhoA and Cdc42 have been shown to be expressed (Fujimoto et al., 2001 and our unpublished results) so the lack of an effect of siRNA against the other Rho GTPases could be due to the absence of, or low expression.

The roles of Rac1 and Cdc42 in phosphorylation of PAK1 and PAK2 following HGF stimulation were investigated. In DU145 cells, both Cdc42 and Rac1 knockdown decreased PAK1 or PAK2 phosphorylation at S199/204 following addition of HGF. PAK1 expression is lower in PC3 cells than DU145 cells and the level of phosphorylated PAK1 detected in these experiments was too low to observe an effect caused by Rac1 knockdown but Cdc42 knockdown slightly impaired phosphorylation of PAK1 and PAK2 at S199/204. In each condition, phosphorylation of PAK1 or PAK2 was not completely blocked, probably because of the presence of other Rho GTPases which can activate PAK, for example Cdc42 can still activate PAK1/2 in Rac1-depleted cells and may compensate for loss of Rac1. It would be interesting to include Rac2 in the assays and use simultaneous knockdown of Rho GTPases to investigate whether this would block PAK activation more effectively. It would also be useful to investigate PAK1 or PAK2 activation using an *in vitro* kinase assay to determine the effect of PAK2 knockdown on PAK1 kinase activity and observe how phosphorylation at S199/204 compares to kinase activity.

In summary, PAK1 and PAK2 are phosphorylated downstream of HGF in DU145, PC3, MCF7 and HT29 cells, and knockdown of Cdc42 or Rac1 impairs this phosphorylation in DU145 and PC3 cells. PAK1 phosphorylation at S199/204 was up-regulated in PAK2-deficient cells suggesting a negative regulation of PAK1 by PAK2 via an as yet unknown mechanism.

Chapter 4: Role of PAK in HGF-induced migration and invasion**4.1: Introduction**

The hepatocyte growth factor (HGF) receptor c-Met is often overexpressed or mutated in cancer, and HGF signalling is involved in many cell processes associated with cancer progression and poor prognosis such as cell migration, invasion, proliferation and angiogenesis (reviewed in Maulik et al., 2002). Growing evidence implicates c-Met and associated signalling pathways as potential therapeutic targets for anti-cancer drugs. For example, HGF stimulates *in vitro* invasion through Matrigel (Khoury et al., 2005) and silencing of c-Met expression in GTL16 gastric carcinoma cells leads to decreased invasion *in vitro*, as well as decreased tumour growth and fewer experimental metastases *in vivo* (Corso et al., 2008). Metastasis of cancer, where single cells escape from the primary tumour and migrate to distal sites, is associated with poor prognosis. *In vitro*, HGF can be used to stimulate cell motility and junctional breakdown mimicking an early stage of metastasis. HGF stimulates scattering of MDCK cells as well as some cancer cell lines including DU145 cells (Miura et al., 2001; Stoker and Perryman, 1985). Scattering is accompanied by internalisation of E-cadherin and a slight decrease in E-cadherin protein levels although it remains expressed.

In keratinocytes, active Rac1 induces junctional breakdown and loss of E-cadherin in a process rescued by knockdown of PAK1 (Lozano et al., 2008). If PAK1 proves to be important for junctional breakdown following HGF stimulation, it would provide further evidence for the pathological significance of PAK1 upregulation in cancer. PAK1 is activated downstream of HGF in MDCK cells and expression of the N-terminal regulatory domain of PAK2 as a dominant negative blocks HGF-induced peripheral actin remodelling and lamellipodium extension (Royal et al., 2000). PAK4 is also activated downstream of HGF in MDCK cells (Wells et al., 2002), and the finding that PAK1 and PAK2 are phosphorylated downstream of HGF in several cancer cell lines (Chapter 3) suggests that PAKs could play a role in the response of cancer cells to HGF.

As well as single cell migration, cells move collectively in groups. This is important during organism development and tissue repair, and it is also the means used by many tumours to invade surrounding tissue (reviewed in (Friedl et al., 2004)). During collective cell migration, the leading cells become polarised with membrane protrusions at the front while

following cells maintain cell junctions and remain attached to the leading cells. Collective cell migration in cancer, also called cohort migration, is stimulated by HGF in several cancer cell lines including HT29 colon cancer cells (Nabeshima et al., 1998) indicating that it could be another mechanism by which up-regulated c-Met signalling leads to poor prognosis. Experimentally, a frequently used method of measuring collective cell migration on a two dimensional substrate is the *in vitro* wound healing assay. This involves scratching a confluent monolayer of cells with a pipette tip and measuring change in wound area over time (Rosello et al., 2004; Valster et al., 2005). In MDCK cells, expression of neither kinase-dead nor constitutively active PAK1 affected the speed of wound healing, but both constructs caused scattering of unstimulated cells and increased migration towards HGF in Boyden chambers (Zegers et al., 2003). This could be due to the ability of both mutants to sequester binding proteins although it is surprising that an increase in migration was observed, as the opposite was true in HMEC-1 mammary epithelial cells (Kiosses et al., 1999).

As well as cell migration, cancer metastasis involves invasion through the extracellular matrix (ECM). PAK1 activity has been shown to correlate with increased invasiveness of breast cancer (Vadlamudi et al., 2000) and expression of kinase dead PAK1 inhibited invasiveness of MDA-MB435 breast cancer cells (Adam et al., 2000). PAK1 knockdown also decreased the invasion of melanocytes through Matrigel, although only one siRNA oligomer was tested (Pavey et al., 2006). HGF regulates cancer cell invasion and has been shown to increase invasion of DU145 cells through Matrigel (Nishimura et al., 1998). There is therefore a case for investigating the roles of PAK isoforms in this process.

The aim of this chapter is to investigate the effect of PAK1, PAK2 or PAK4 depletion on the response of cancer cells to HGF.

4.2: The response of cancer cells to HGF

DU145 cells scatter in response to HGF (Miura et al., 2001) and groups of HT29 cells become more spread (Nabeshima et al., 1998). In chapter 3, PAK1 and PAK2 were shown to become phosphorylated following HGF stimulation in DU145, PC3, MCF7 and HT29 cells. In order to set up an assay to investigate the functional significance of PAK1, PAK2 and PAK4 downstream of HGF in these cancer cell lines, the response of sub-confluent

cells to HGF was observed. DU145, MCF7 and HT29 cells grow in groups with epithelial morphology so movies were made to test for scattering or increased spreading (Figure 4.1). PC3 cells grow as single cells and so do not scatter so their response was assayed separately. The previously described scattering response of DU145 cells in serum-starved conditions was reproduced, whereas MCF7 cells did not scatter and exhibited only a weak response to HGF by increased ruffling at early time points, and no significant response could be observed in HT29 cells.

Because DU145 cells responded more strongly to HGF than MCF7 and HT29 cells, they were chosen as a model to investigate the role of PAK knockdown on HGF-induced scattering. The DU145 cell scattering assay was optimised by testing varying concentrations of HGF in 10% or 0.1% FCS (Figure 4.2). There was no difference in scattering between 10 ng/ml or 20 ng/ml HGF and cells scattered in 10% or 0.1% FCS although at later time points, cell death was observed in 0.1% FCS (24 hour time point not shown). The conditions used for further assays were 10 ng/ml HGF in 10% FCS.

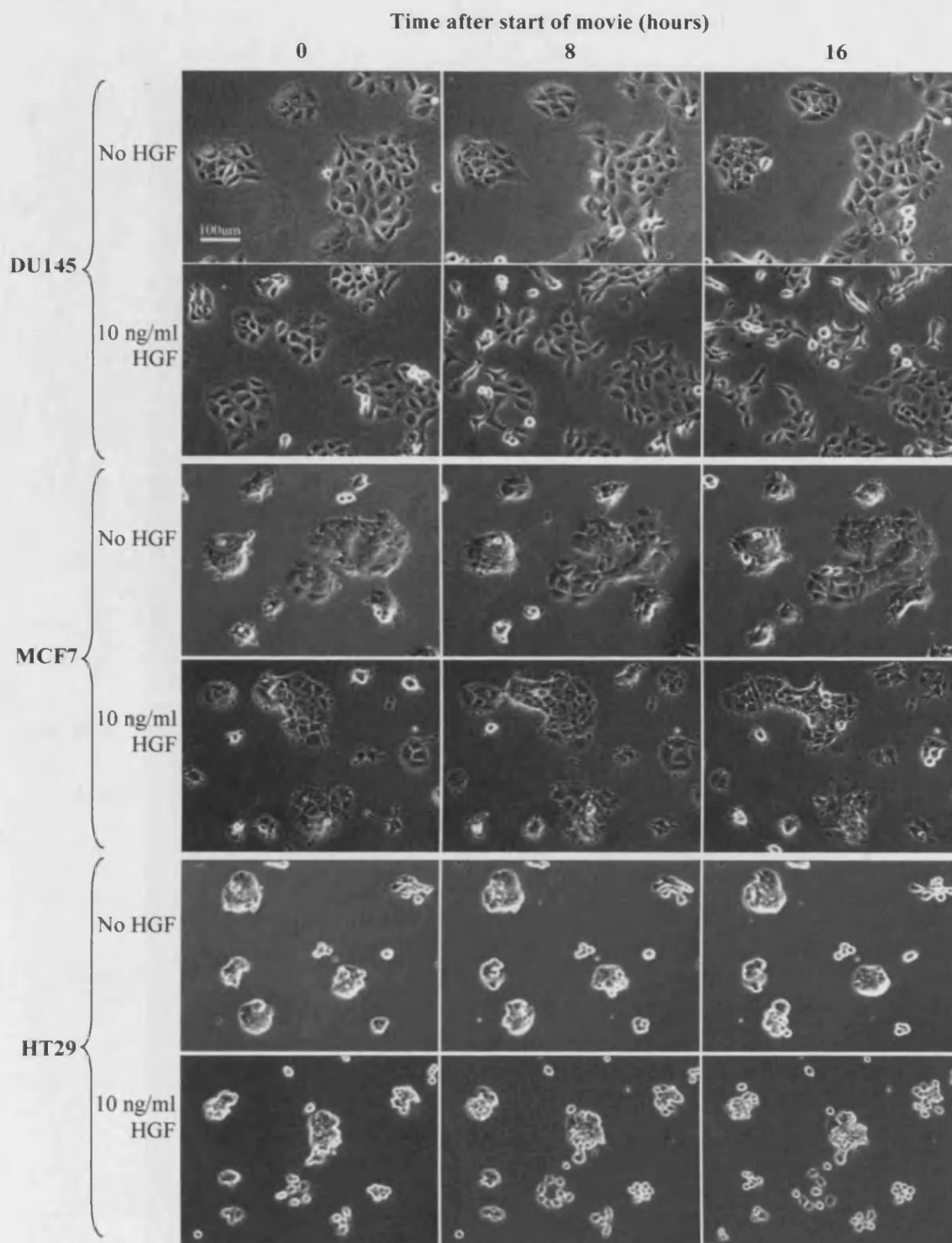


Figure 4.1: The response of DU145, MCF7 and HT29 cells to HGF. Cells were seeded and allowed to grow for 48 hours prior to serum starvation in 0.1% FCS for 6 hours. Cells were then stimulated with 10 ng/ml HGF or kept in starved conditions and movies were made for 16 hours taking one frame every 10 minutes. T=0 corresponds to the start of the movie 30 minutes after HGF addition. Data are representative of at least 2 experiments.

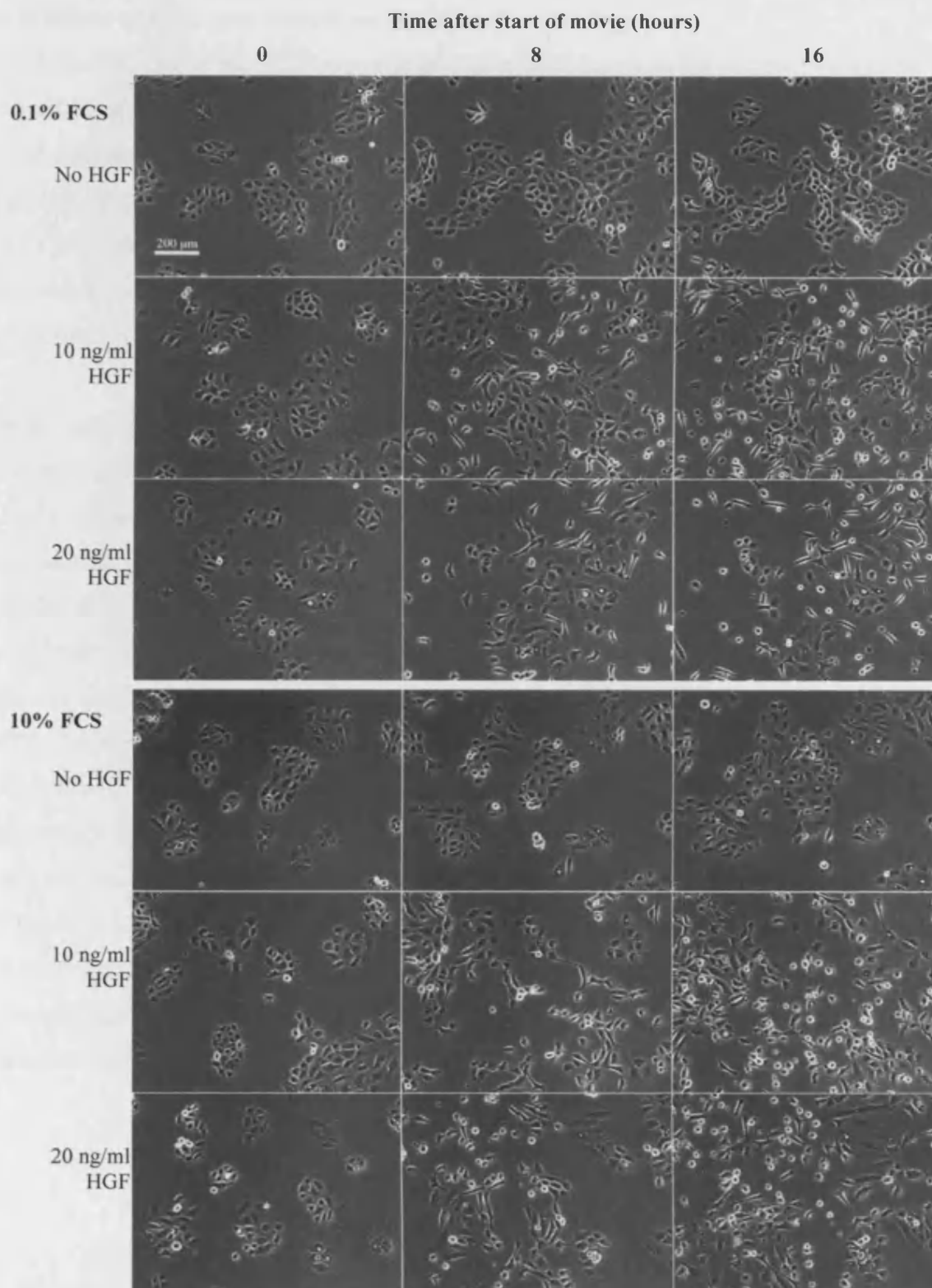


Figure 4.2: Optimization of the DU145 cell scattering assay. DU145 cells were seeded and allowed to grow for 48 hours. Cells were either kept in growth medium or serum starved in 0.1% FCS for 6 hours prior to stimulation with 0, 10 or 20 ng/ml HGF. Movies were made for 24 hours taking one frame every 10 minutes (0 – 16 hours shown). T=0 corresponds to the start of the movie 30 minutes after HGF addition.

4.3: Effect of PAK knockdown on DU145 cell scattering

The role of PAKs in the HGF response of cancer cell lines is as yet unclear. To address this, the effect of PAK1, PAK2 or PAK4 depletion by RNAi on DU145 cell scattering following HGF stimulation was observed. Movies were made for 24 hours taking one frame every 5 minutes (supplementary movies 1-14), or every 10 minutes in subsequent experiments. Four separate oligomers were tested for each isoform to control for non-specific effects of individual siRNAs. A control oligomer with no known target was used and no difference was observed between this and untransfected cells.

In the case of PAK1, the oligomer siPAK1a had no detectable effect and cells scattered in the same way as control cells. However, the other three of four oligomers used against PAK1 caused fewer cells to dissociate from groups and become rounded following HGF stimulation compared to control cells (Figure 4.3 and Supplementary movies 1-6). The results did not match exactly with the efficiency of knockdown observed for each oligomer. Previous western blotting showed that each PAK1 oligomer knocked down protein expression but oligomer PAK1b was least efficient (Figure 3.9). siPAK1c and siPAK1d were chosen for future experiments due to their similar knockdown efficiency and phenotype. Their effect on cell rounding in response to HGF was quantified and found to be significant for oligomer PAK1d (Figure 4.12). The localisation of actin filaments and E-cadherin in DU145 cells before and after 18 hours of HGF stimulation was observed (Figures 4.4 and 4.5). HGF stimulation led to loss of E-cadherin from junctions as expected in control cells but this was retained in the junctions of PAK1-depleted DU145 cells. Actin filament distribution appeared similar in PAK1-depleted cells. However, more rounded, detached cells were observed when control siRNA was used as in time lapse movies.

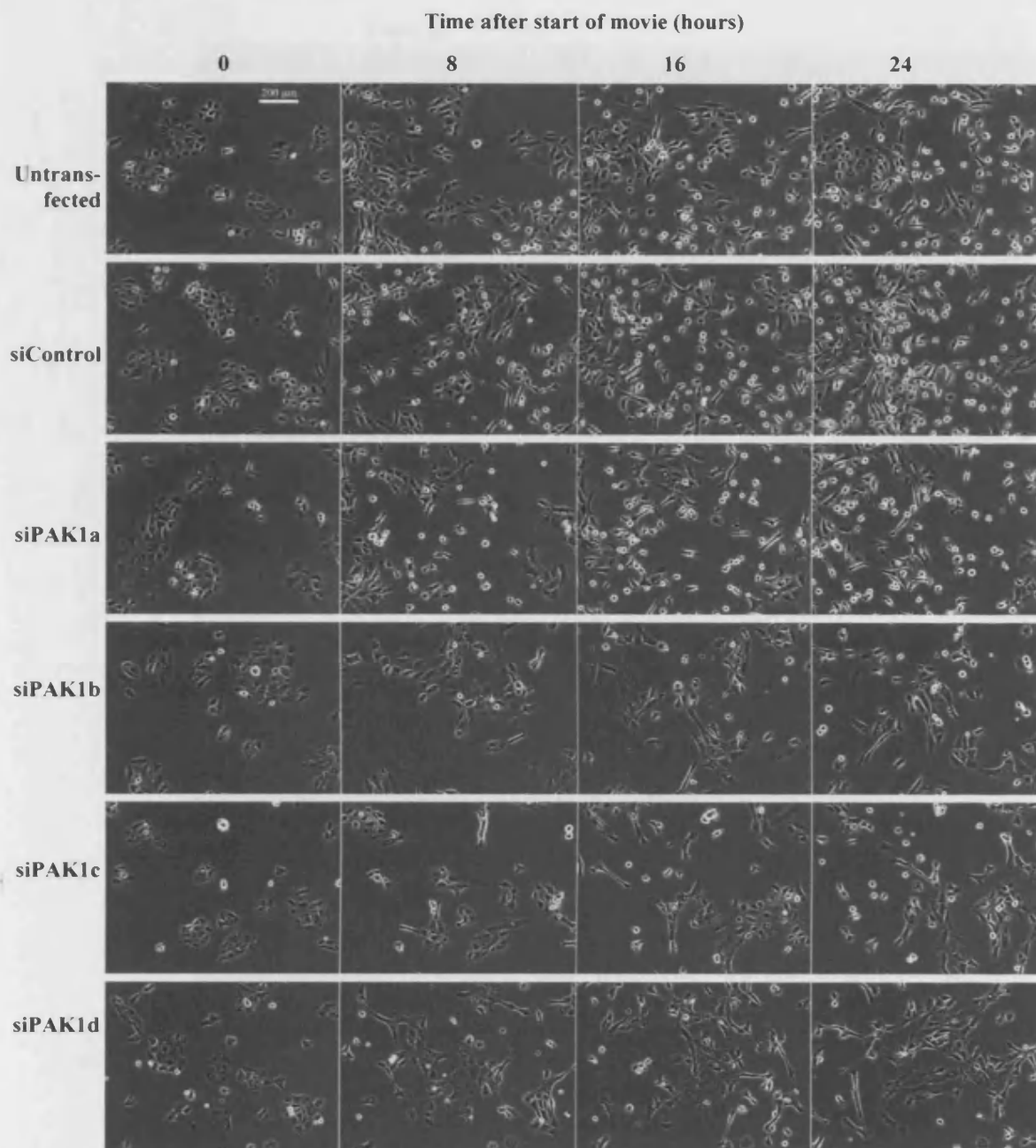


Figure 4.3: PAK1 knockdown impairs scattering of DU145 cells. DU145 cells were seeded and allowed to grow for 16 hours before transfection with control siRNA or one of four different siRNA oligomers against PAK1. 48 hours after transfection cells were stimulated with 10 ng/ml HGF and movies were made for 24 hours taking one frame every 5 minutes. T=0 corresponds to the start of the movie approximately 30 minutes after HGF stimulation. Movies are representative of at least four repeat experiments for untransfected, siControl, siPAK1c and siPAK1d, and one experiment for siPAK1a and siPAK1b.

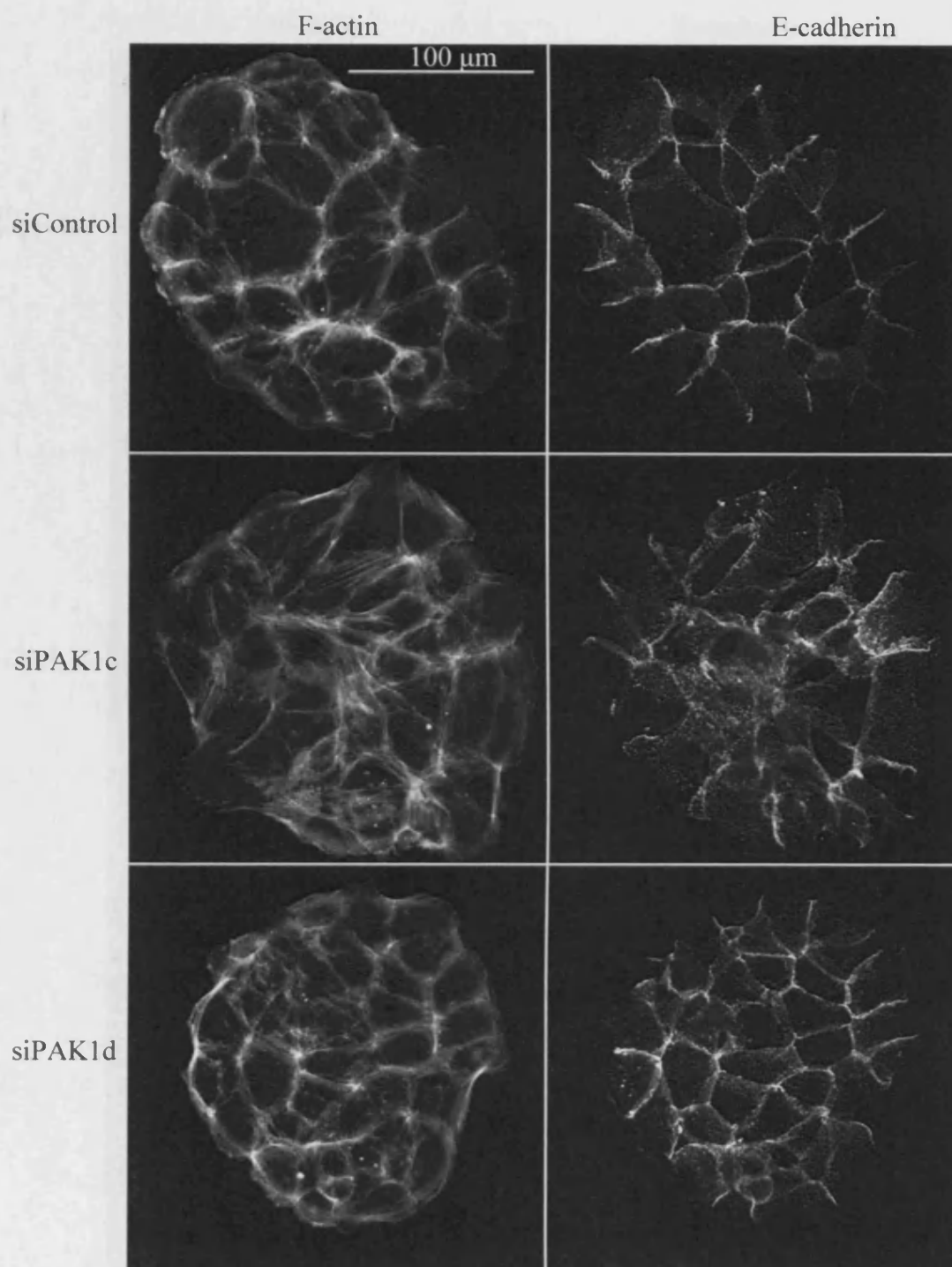


Figure 4.4: Actin filament and E-cadherin localisation in unstimulated DU145 cells. DU145 cells were treated with control siRNA or one of two oligomers of siRNA against PAK1. After 66 hours cells were fixed in 4% PFA and stained for actin filaments with fluorescent phalloidin (F-actin) or E-cadherin with an anti E-cadherin antibody.

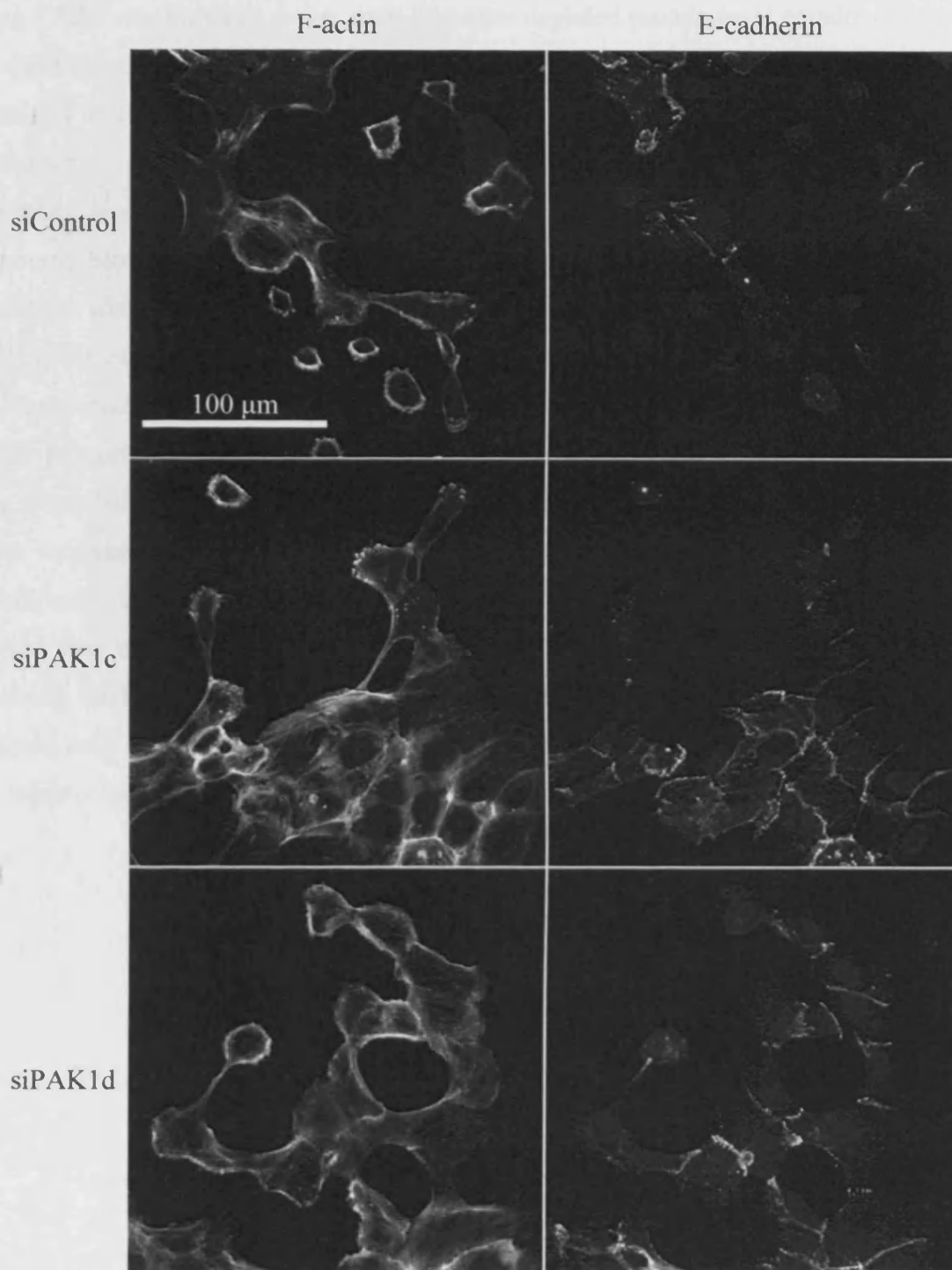
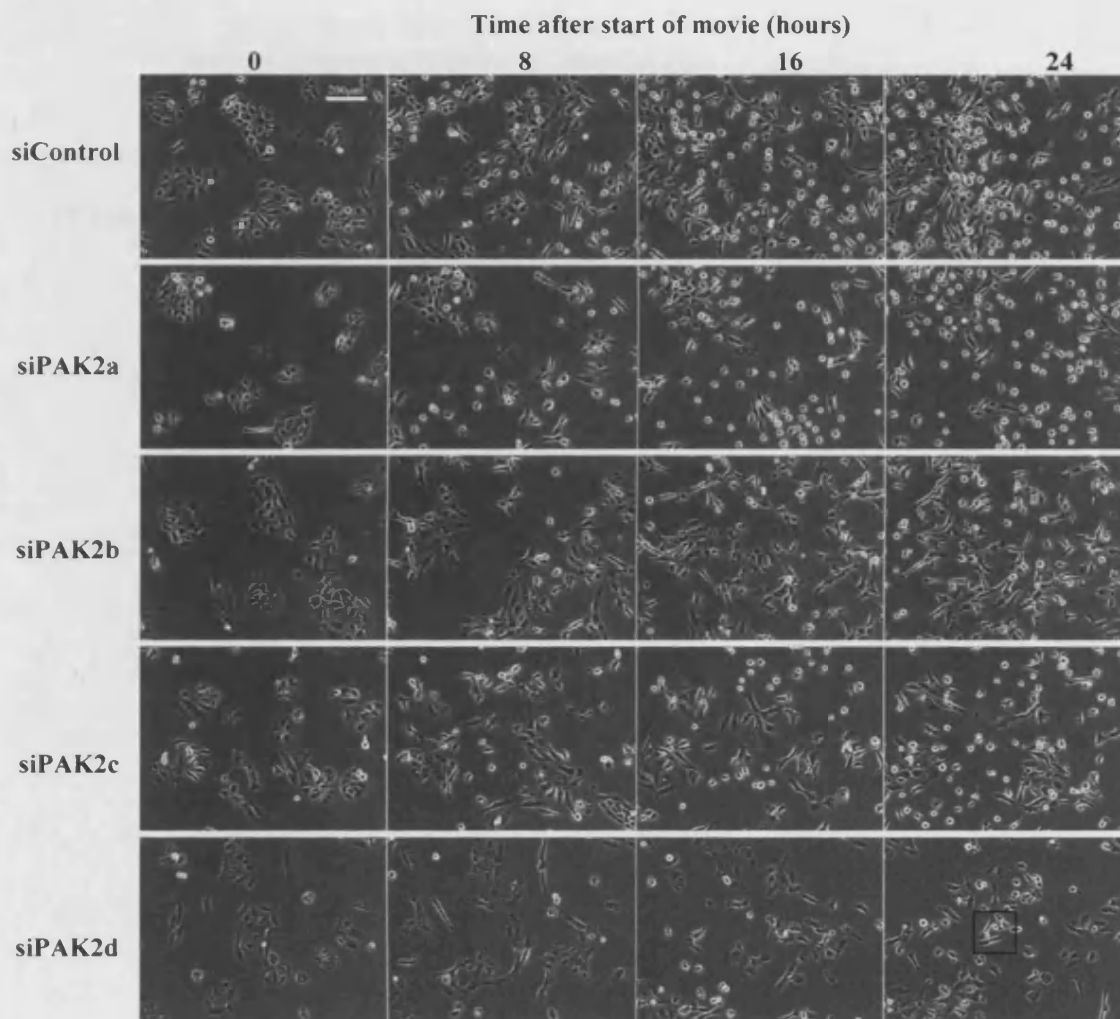


Figure 4.5: E-cadherin persists in junctions in PAK1-depleted DU145 cells following HGF treatment. DU145 cells were treated with control siRNA or one of two oligomers of siRNA against PAK1. After 48 hours cells were stimulated with 10 ng/ml HGF for 18 hours, then fixed in 4% PFA and stained for actin filaments with fluorescent phalloidin (F-actin) or E-cadherin with an anti-E-cadherin antibody.

When PAK2 was knocked down, each oligomer depleted protein level equally (Figure 3.9) but there was again some variation in the phenotype observed. Oligomer siPAK2a did not detectably affect scattering (Figure 4.6 and supplementary movie 7) and oligomer siPAK2d caused some cells to bleb (Figure 4.6B and supplementary movie 10). The remaining two oligomers were chosen for future experiments because they gave the most similar phenotype although there was still a difference. Oligomer siPAK2b gave the strongest phenotype where cells rounded up less although it was not statistically significant (Figure 4.12). Cells treated with oligomer siPAK2b also had larger lamellipodia (Figure 4.9, 4.10 and supplementary movie 8). When oligomer siPAK2c was used cells scattered and a similar proportion rounded compared to control conditions but a subset of cells appeared to have larger lamellipodia similar to siPAK2b (Figure 4.7, 4.10 and supplementary movie 9). These experiments should be repeated to determine whether the difference in size of lamellipodia is statistically significant. Confocal microscopy and staining for F-actin showed that knockdown of PAK2 led to broader lamellipodia in a proportion of cells following HGF stimulation (Figure 4.9). Prior to HGF stimulation, some groups of PAK2-depleted cells appeared less stable than control cells. Gaps between junctions were visible and some cells extended away from colonies (Figure 4.8 arrows).



B siPAK2d 24 hours HGF

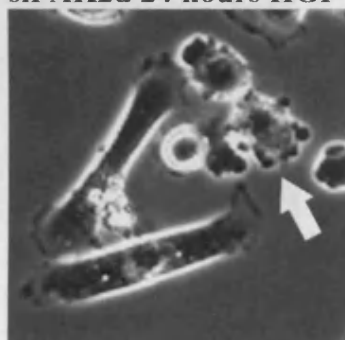


Figure 4.6: PAK2-depleted DU145 cells remain more spread following HGF stimulation. A) DU145 cells were seeded and allowed to grow for 16 hours before transfection with control siRNA or one of four different siRNA oligomers against PAK2. 48 hours after transfection cells were stimulated with 10 ng/ml HGF and movies were made for 24 hours taking one frame every 5 minutes. T=0 corresponds to the start of the movie 30 minutes after HGF stimulation. Movies are representative of at least four repeat experiments for siControl, siPAK2b and siPAK2c, only one experiment was performed for siPAK2a and siPAK2d. B) 4X magnification of boxed region in siPAK2d 24 hour timepoint showing blebbing (arrow).

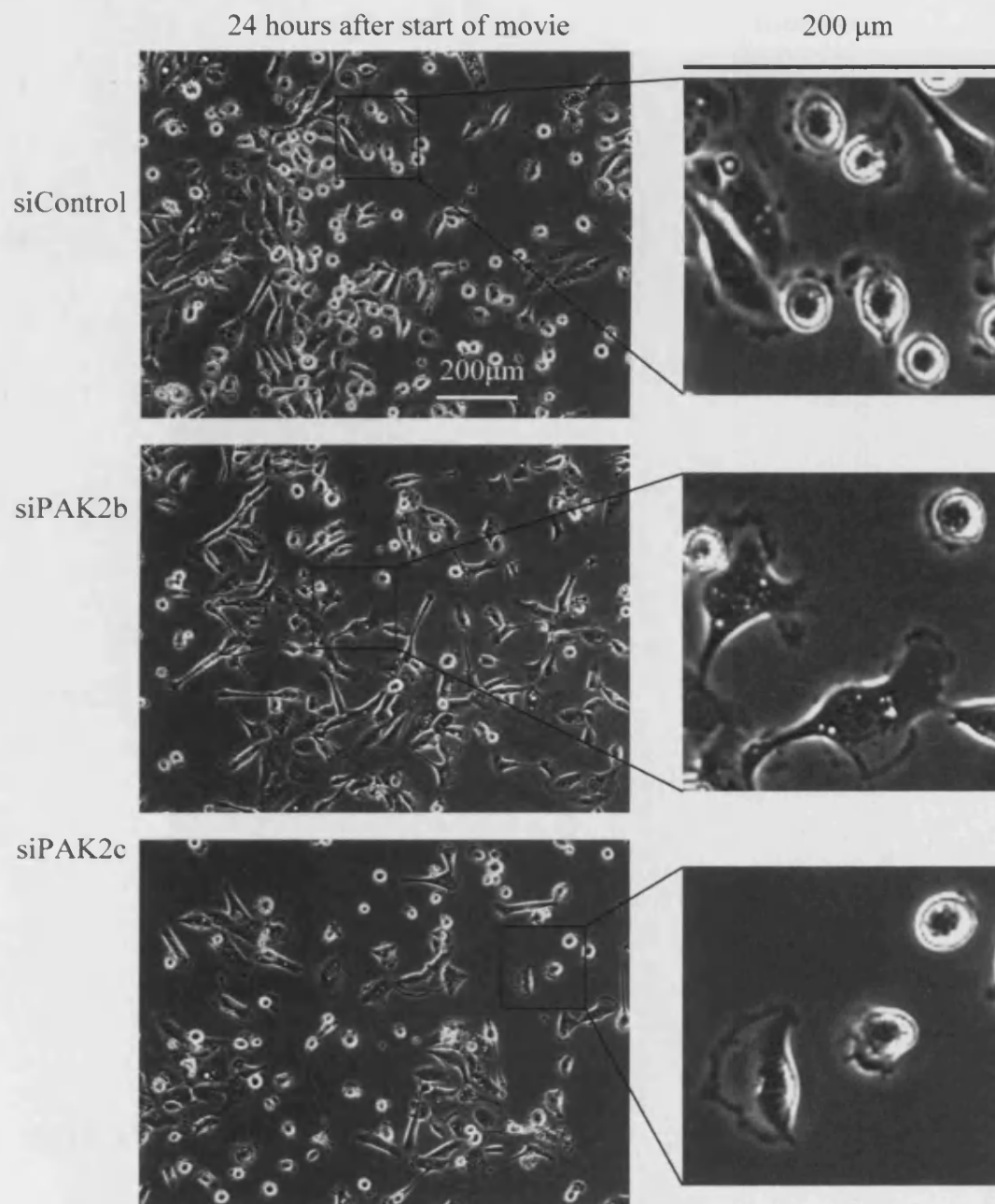


Figure 4.7: PAK2 knockdown in DU145 cells affects lamellipodia. Details from 24 hour timepoints from figure 4 were magnified digitally to 4X magnification.

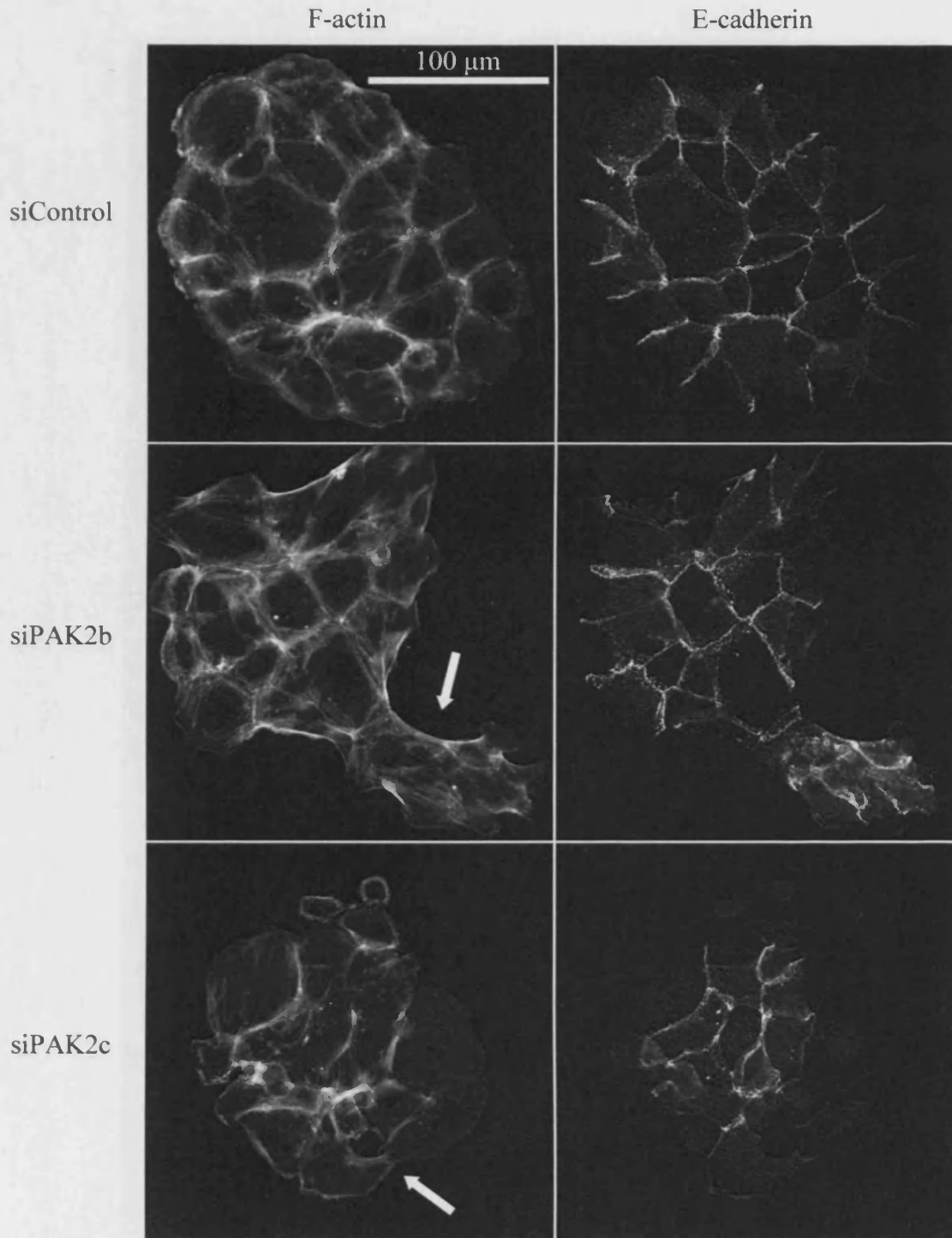


Figure 4.8: Effect of PAK2 knockdown on morphology of DU145 cell colonies. DU145 cells were treated with control siRNA or one of two oligomers of siRNA against PAK2. After 66 hours cells were fixed in 4% PFA and stained for actin filaments with fluorescent phalloidin (F-actin) or E-cadherin with an anti-E-cadherin antibody. Note that control images are the same as in figure 4.4 as they are from the same experiment. Arrows show where there are gaps in colonies (siPAK2c) or where cells appear to be pulling away from groups (siPAK2b).

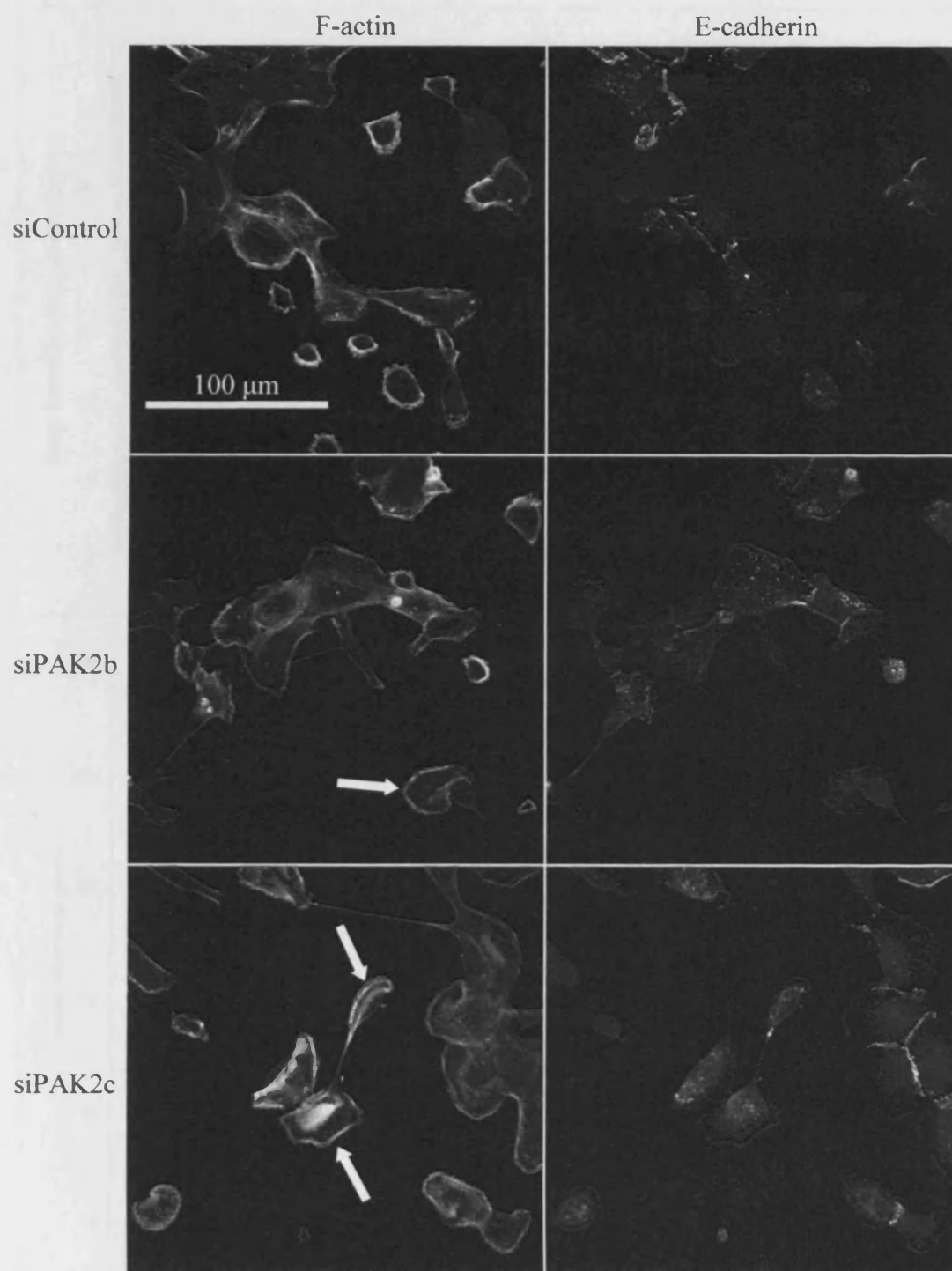


Figure 4.9: Actin staining in lamellipodia is different in PAK2 deficient DU145 cells following HGF stimulation. DU145 cells were treated with control siRNA or one of two oligomers of siRNA against PAK2. After 48 hours cells were stimulated with 10 ng/ml HGF for 18 hours, then fixed in 4% PFA and stained for actin filaments with fluorescent phalloidin (F-actin) or E-cadherin with an anti-E-cadherin antibody. Note that control images are the same as in figure 4.5 as they are from the same experiment.

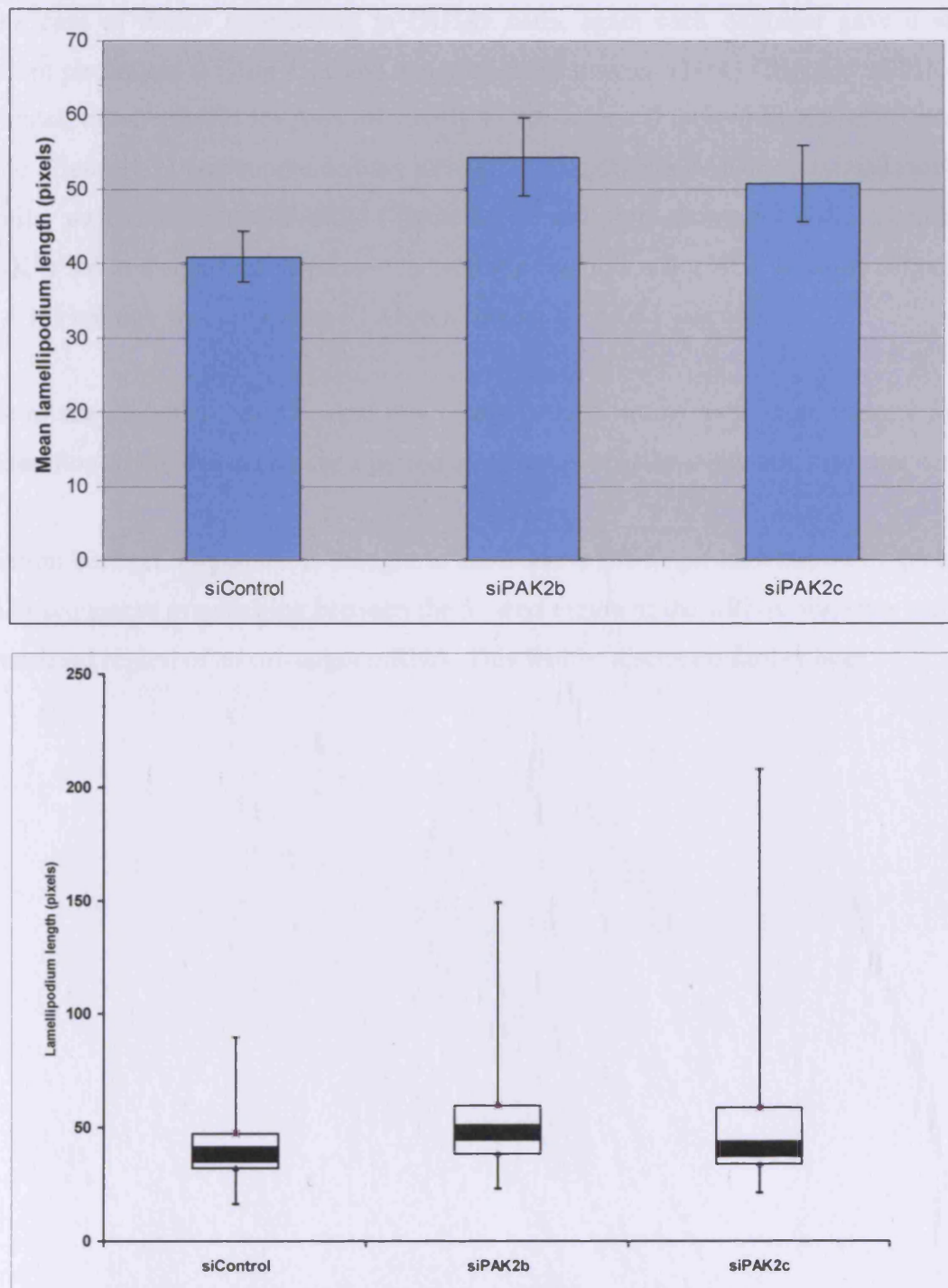
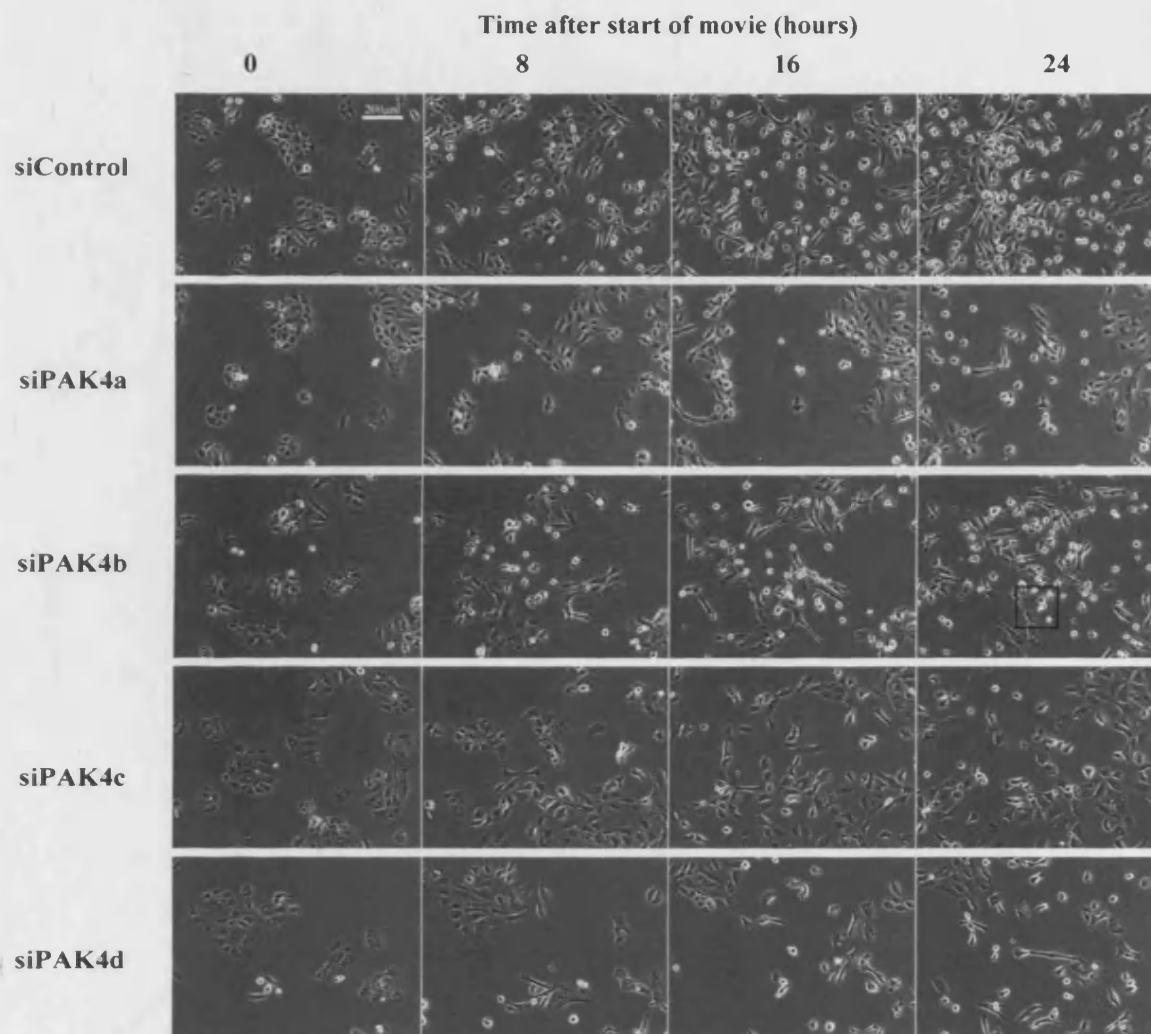


Figure 4.10: PAK2 knockdown leads to larger lamellipodia in DU145 cells following HGF stimulation. The length of lamellipodia in control or PAK2-depleted cells following HGF stimulation for 24 hours was measured. Cells which were not in contact with more than one other cell and also had visible lamellipodia were selected and the length of the leading edge of lamellipodia was measured. Data are expressed as average lamellipodium length (top) or as a box and whisker diagram representing at least 40 cells for each siRNA condition over three independent experiments. T-tests did not reveal statistical significance.

In the case of PAK4 knockdown in DU145 cells, again each oligomer gave a slightly different phenotype (Figure 4.11 and supplementary movies 11-14). Oligomer siPAK4a did not knock down protein level as efficiently as the others (Figure 3.9) and cells remained motile (Figure 4.11 and supplementary movie 11). Oligomer siPAK4b cause cells to bleb in a similar way to oligomer siPAK2d (Figure 4.11B and supplementary movie 12). Oligomer siPAK4c led to a significant decrease in cell rounding following HGF addition (Figure 4.11 and 4.12) but this was not observed when oligomer siPAK4d was used.

None of the oligomers used against any of the PAK isoforms tested significantly reduced proliferation of DU145 cells over a period of 24 hours of HGF stimulation (Figure 4.13).

Variation between oligomers is thought to arise due to off-target knockdown of genes with similar sequences or matching between the 5' seed region of the siRNA oligomer and the 3' untranslated region of an off-target mRNA. This will be discussed further later.



B siPAK4b 24 hours HGF

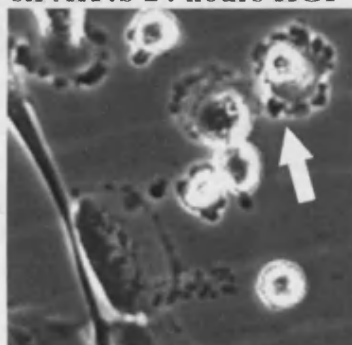


Figure 4.11: Knockdown of PAK4 in DU145 cells. DU145 cells were seeded and allowed to grow for 16 hours before transfection with control siRNA or one of four different siRNA oligomers against PAK4. 48 hours after transfection cells were stimulated with 10 ng/ml HGF and movies were made for 24 hours taking one frame every 5 minutes. T=0 corresponds to the start of the movie approximately 30 minutes after HGF stimulation. Movies are representative of at least four repeat experiments for siControl, siPAK4c and siPAK4d, only one experiment was performed for siPAK4a and siPAK4b. B) 4X magnification of boxed region in siPAK4b, 24 hour timepoint, showing blebbing (arrow).

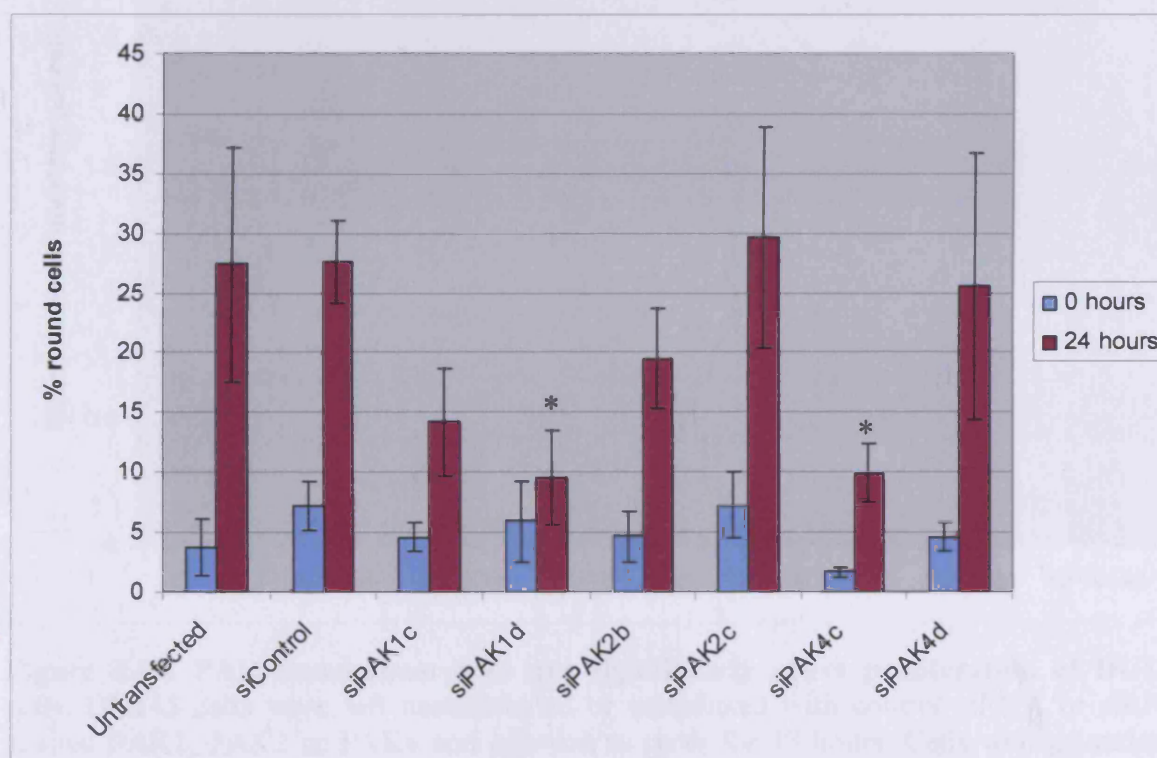


Figure 4.12: PAK1 knockdown decreases cell rounding in response to HGF. DU145 cells were left untransfected or transfected with control siRNA or siRNA against PAK1, PAK2 or PAK4 and allowed to grow for 48 hours. Cells were stimulated with 10 ng/ml HGF and movies were made for 24 hours. The first and last frames were taken and rounded cells were counted automatically using Metamorph software as described in Chapter 2. Round cells were expressed as a percent of the total cell number. Graphs include data from 3 separate experiments. Error bars represent S.E.M., * = $p < 0.05$, two tailed t test.

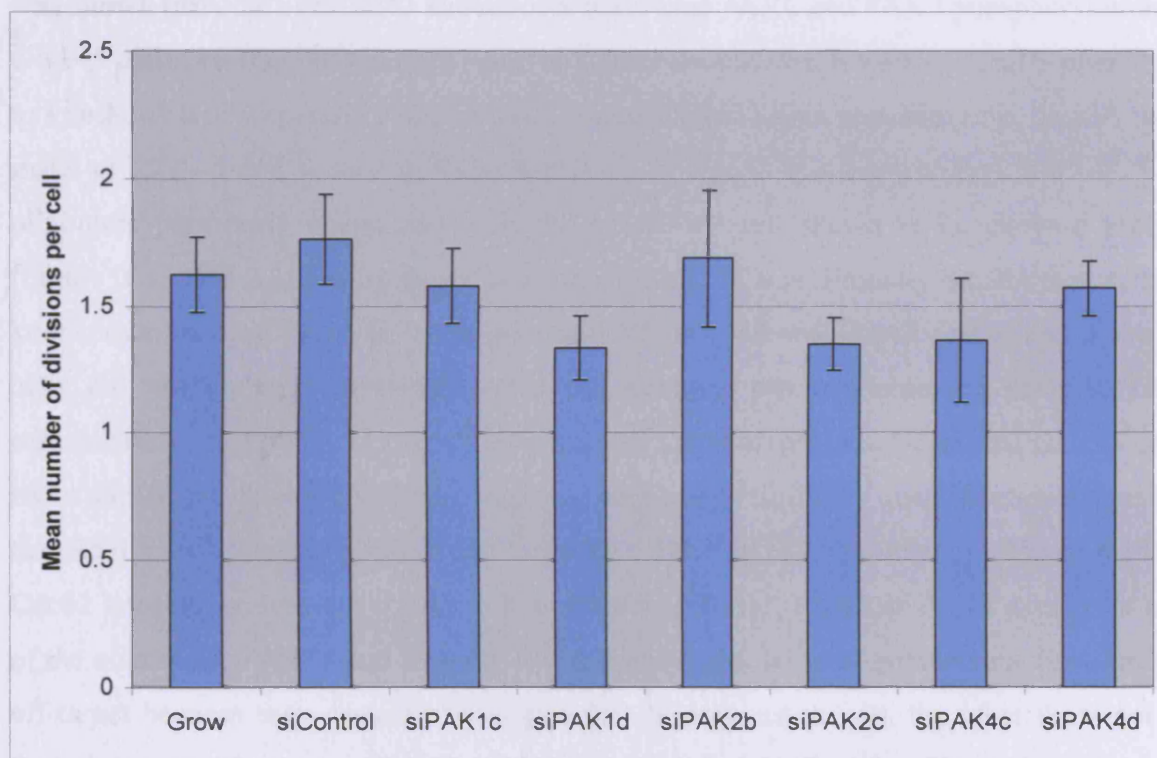
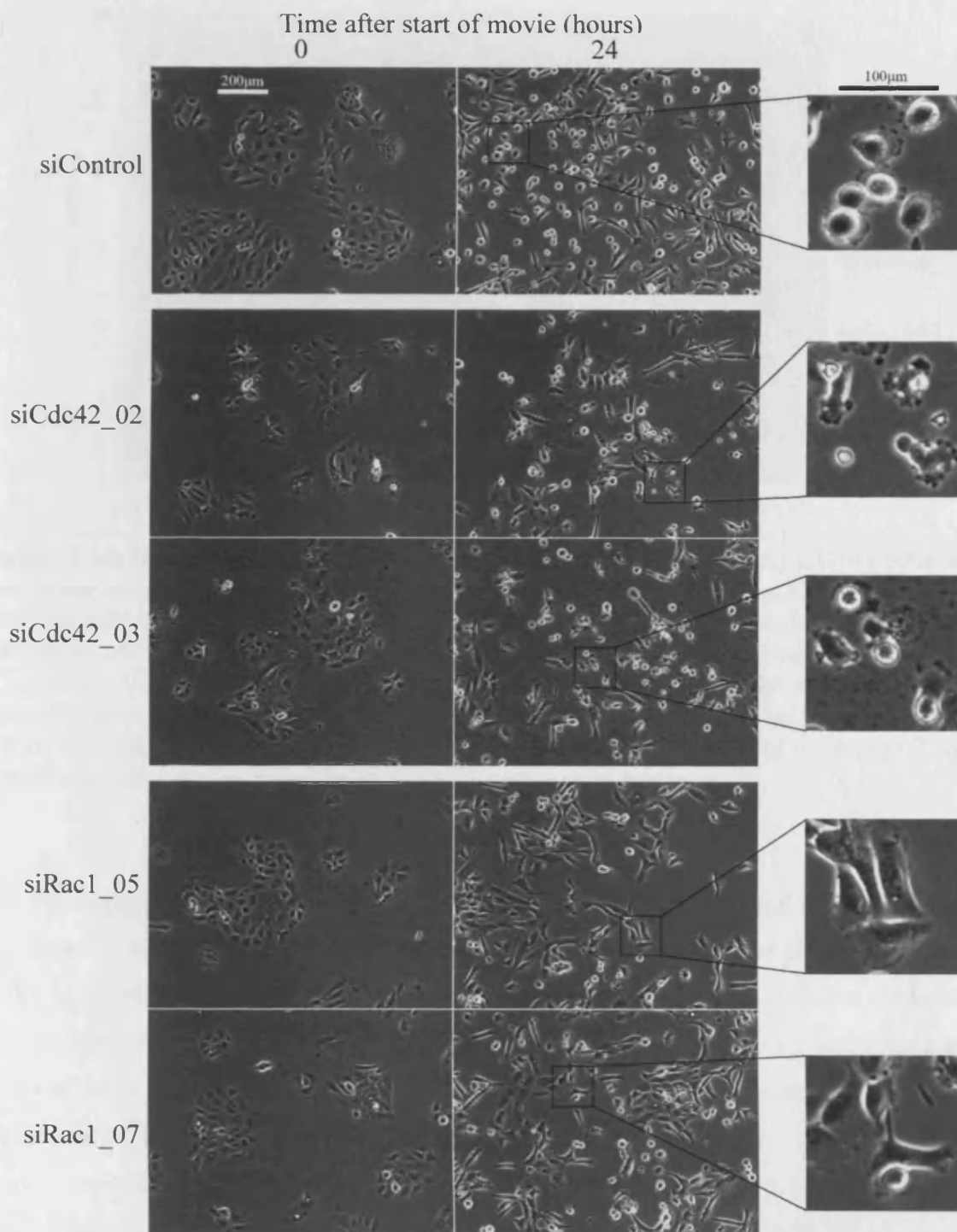


Figure 4.13: PAK knockdown does not significantly affect proliferation of DU145 cells. DU145 cells were left untransfected or transfected with control siRNA or siRNA against PAK1, PAK2 or PAK4 and allowed to grow for 48 hours. Cells were stimulated with 10 ng/ml HGF and movies were made for 24 hours. The total number of cells in the last frame of each movie was divided by the total number of cells in the first frame to give an approximate number of cell divisions. The mean number of cell divisions over three independent experiments is shown. Error bars represent S.E.M. T-tests did not reveal statistical significance.

4.4: Effect of Rho GTPase knockdown on DU145 cell scattering

PAK activity and localisation can be controlled by binding of active Rho GTPases. Rac1 and Cdc42 in particular have been linked to activation of PAK1 and PAK2. In chapter 3 it was shown that Rac1 or Cdc42 knockdown decreased PAK1 and PAK2 phosphorylation in DU145 cells. To observe whether Rac1 or Cdc42 knockdown leads to a similar phenotype to knockdown of a specific PAK isoform, hence suggesting an upstream role, movies were made of DU145 cell scattering following Rac1 or Cdc42 knockdown (Figure 4.14). Two oligomers previously characterised in the laboratory and shown to knockdown protein (Figure 3.15 and 3.16) were used for each GTPase. In a preliminary experiment, Cdc42 knockdown induced blebbing in response to HGF but cells could still scatter and blebbing cells did not undergo apoptosis. Increased blebbing was not observed prior to HGF stimulation. In comparison to the PAK knockdown phenotypes, Rac1-depleted DU145 cells had a similar morphology to PAK1-depleted cells and preliminary quantification suggested that Rac1 knockdown may block cell rounding following HGF stimulation (Figure 4.14B). Cdc42 knockdown was not similar to knockdown of PAK1, PAK2 or PAK4 except for two of the oligomers; PAK2d and PAK4b. It was thought that these oligomers may have had an off-target because they caused phenotypes that did not match with the other three out of four oligomers. These experiments need to be repeated to confirm the observed results.

A



B

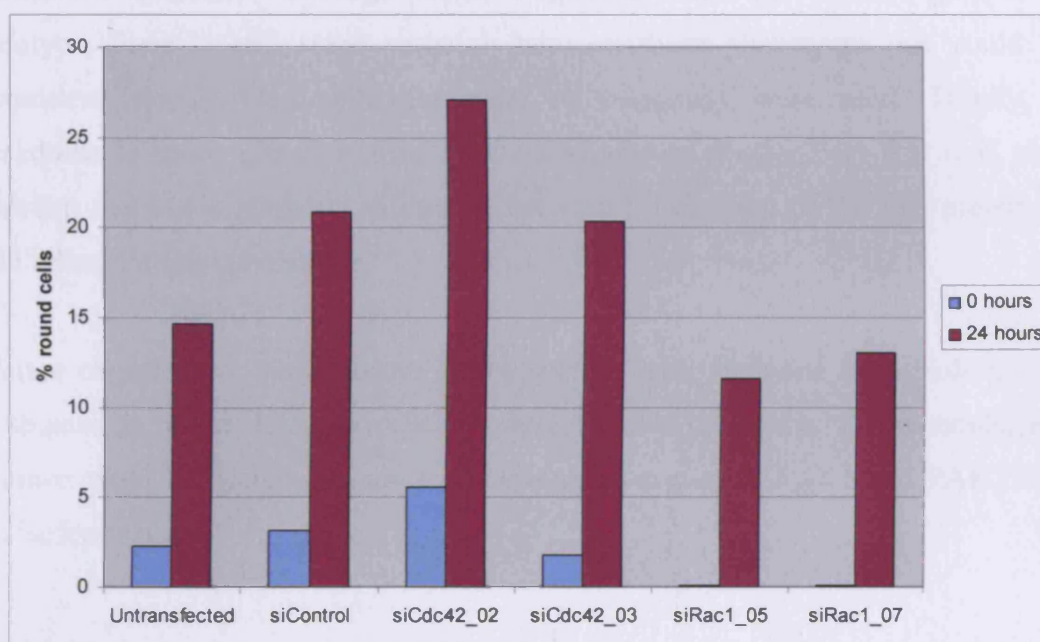


Figure 4.14: Rac1 or Cdc42 knockdown give distinct phenotypes. A) DU145 cells were seeded and allowed to grow overnight before transfection with control siRNA or one of two different siRNA oligomers against Rac1 or Cdc42. 48 hours after transfection, cells were stimulated with 10 ng/ml HGF and movies were made for 24 hours taking one frame every 10 minutes (0 and 24 hour timepoints shown). T=0 corresponds to the start of the movie approximately 30 minutes after HGF stimulation. B) Percent round cells was quantified before and after 24 hours of HGF stimulation according to the protocol in chapter 2 using frames from the movies shown in A. Data are from one experiment.

4.5: Phenotypes of cells following combined knockdown of PAK1 and PAK2

In Chapter 3 it was shown that knockdown of PAK2 causes increased phosphorylation of PAK1 at S199/204. To determine whether the upregulation of PAK1 could be responsible for the phenotypes observed when PAK2 was knocked down, both PAK1 and PAK2 were knocked down simultaneously using two different combinations of oligomers (Figure 4.15). Simultaneous knockdown of PAK1 and PAK2 had been previously tested by western blotting using lysate from DU145 cells and found to be as effective as knocking down single isoforms (result not shown). When siPAK2b was cotransfected with siPAK1d, cells appeared flatter than with siPAK2b alone. However when siPAK2c and siPAK1c were cotransfected, lamellipodia appeared the same as with PAK2c alone. These preliminary results were therefore inconclusive, probably due to a number of factors which could affect the phenotype seen in cells. Firstly, siRNA knockdown of protein is not complete and the phenotype observed may depend on the relative amounts of protein remaining following

knockdown. Secondly, although single oligomers were chosen that gave a similar phenotype, there is still some variation between those phenotypes that could lead to inconsistent results when different pairs of oligomers were used. Thirdly, PAK2 knockdown is more effective than PAK1 knockdown (Figure 3.9) and it is unknown whether there is a temporal difference between knockdown of the two proteins which could affect the final phenotype.

In future experiments, simultaneous knockdown of more than one PAK isoform could be investigated in more detail to produce more conclusive results. For example, a single oligomer could be designed against a homologous region of PAK1 and PAK2 to knock both isoforms down.

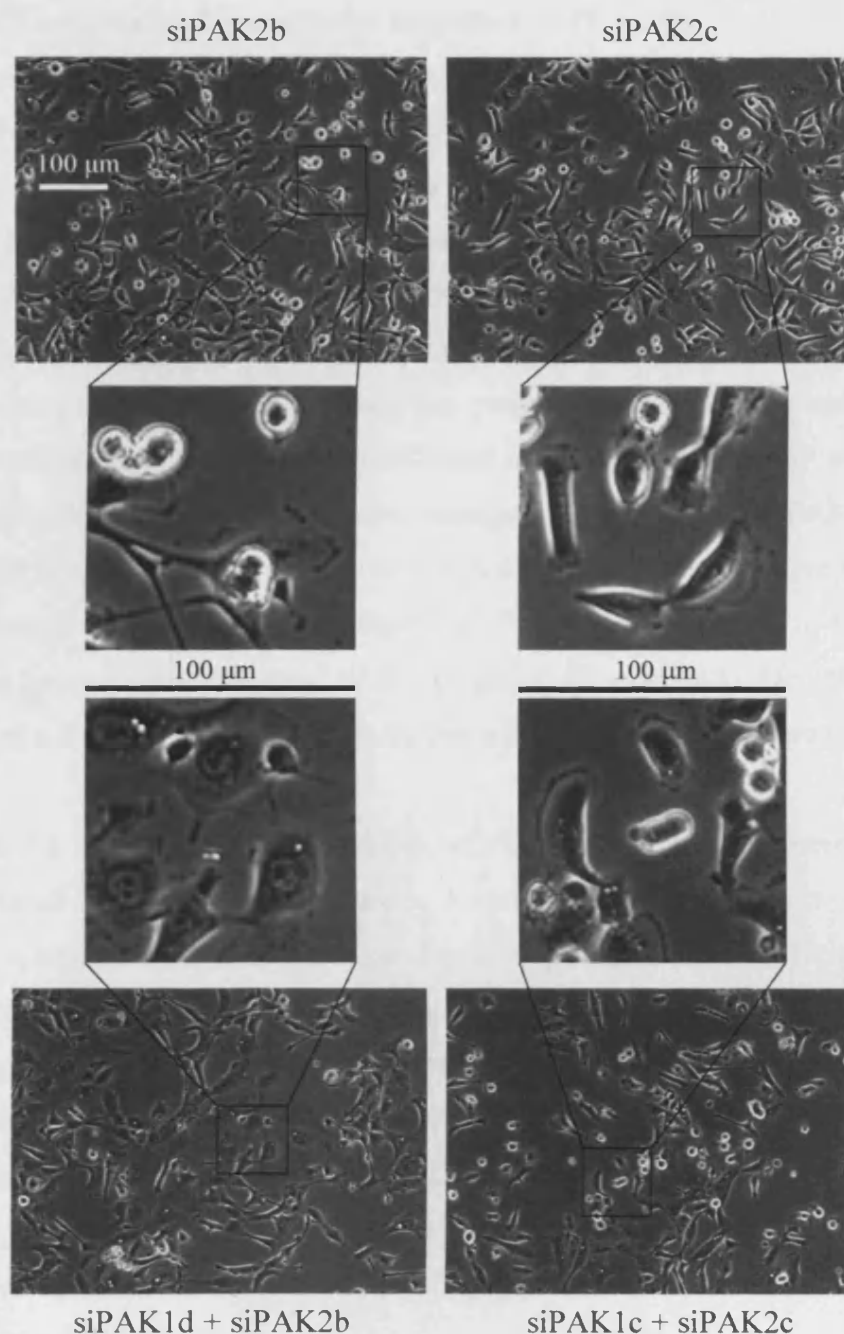


Figure 4.15: PAK1 and PAK2 knockdown simultaneously in DU145 cells. DU145 cells were seeded and allowed to grow overnight before transfection with control siRNA (not shown), one of two different siRNA oligomers against PAK2 or the combinations of PAK1 and PAK2 oligomers indicated. 48 hours after transfection, cells were stimulated with 10 ng/ml HGF and movies were made for 24 hours taking one frame every 10 minutes (24 hour timepoint shown). Boxed areas are magnified fourfold to show detail of cell morphology and lamellipodia. Data are from one experiment

4.6: PAK1 or PAK2 knockdown impairs migration of PC3 cells

PC3 cells were not included in scattering assays because they grow as single cells. They express c-Met and respond to HGF and so they provide a model system to assess the role of PAK isoforms in the migration of single cells. This is relevant to the pathological process by which single cells which have escaped from a tumour mass migrate to distal sites. The effect of knockdown of PAK1, PAK2 or PAK4 on PC3 migration and morphology was assessed in movies for 16 hours taking one frame every 10 minutes (Figures 4.16 – 4.18 and supplementary movies 15-28). Analysis was performed on the first 12 hours of movies due to cell proliferation at later timepoints. Control cells responded to HGF with increased motility (Figure 4.20) and became more elongated (Figure 4.16). PAK1 or PAK4 knockdown did not significantly affect the morphology of PC3 cells before or after HGF treatment although one of the PAK4 oligomers (PAK4b) caused cells to become more elongated than control cells following HGF stimulation (Figure 4.18). Depletion of PAK2 caused more cells to be localised in groups before and after HGF stimulation (Figure 4.17).

To investigate the effect of PAK knockdown on PC3 migration in the presence of HGF, cells were tracked over a period of 12 hours. Knockdown of PAK2 led to cell migration tracks which appeared shorter and less persistent (Figure 4.19). The difference between tracks from control cells and tracks from PAK1 or PAK4-depleted cells was less clear although it appeared that PAK4-depleted cell migration may be more persistent. Persistence should be measured in future to quantify these results. The mean speed of PC3-cell migration in movies was measured (Figure 4.20). Knockdown of PAK1 or PAK2 slowed the average speed of PC3 migration whereas knockdown of PAK4 had no effect on the migration speed compared to control siRNA, although more experiments are required.

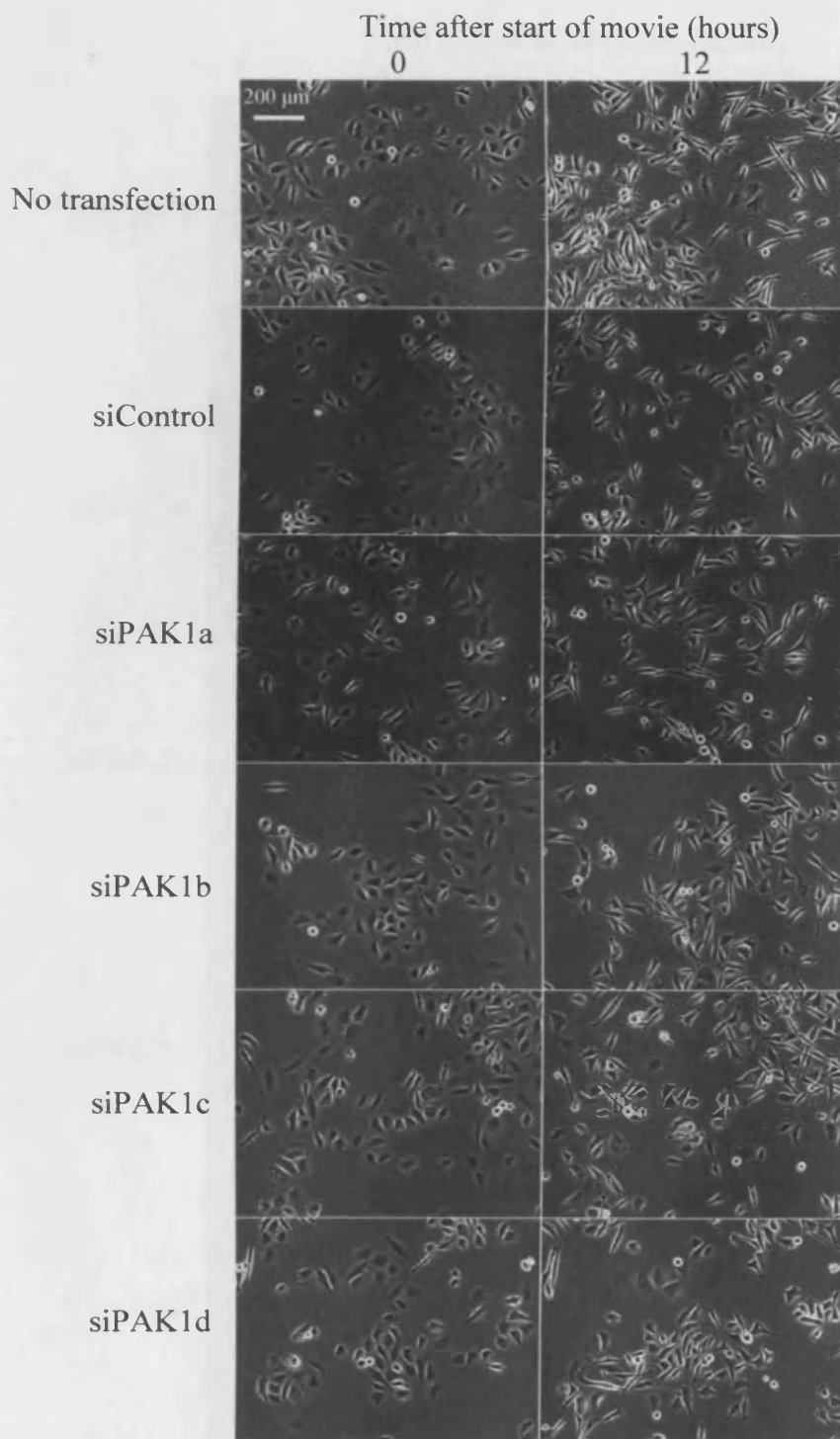


Figure 4.16: PAK1 knockdown does not affect PC3 morphology. PC3 cells were seeded and allowed to grow for 18 hours before transfection with control siRNA or one of four different oligomers against PAK1. Forty-eight hours after transfection, cells were stimulated with 10 ng/ml HGF and movies were made for 16 hours taking one frame every 10 minutes (0 and 12 hour timepoints shown). Movies are representative of two repeat experiments.

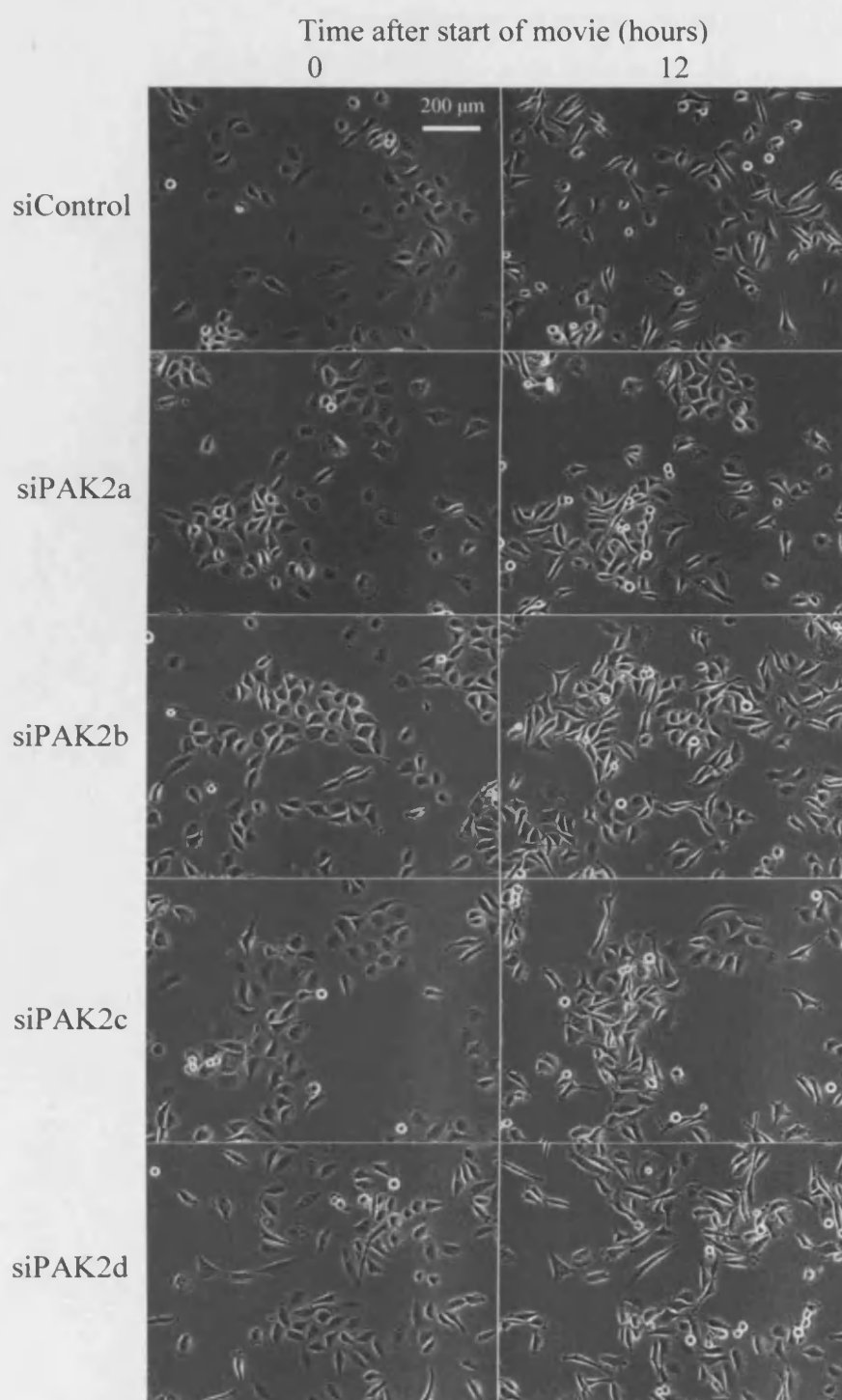


Figure 4.17: PAK2 depleted PC3 cells localise in groups. PC3 cells were seeded and allowed to grow for 18 hours before transfection with control siRNA or one of four different oligomers against PAK2. Forty-eight hours after transfection, cells were stimulated with 10 ng/ml HGF and movies were made for 16 hours taking one frame every 10 minutes (0 and 12 hour timepoints shown). Movies are representative of two repeat experiments.

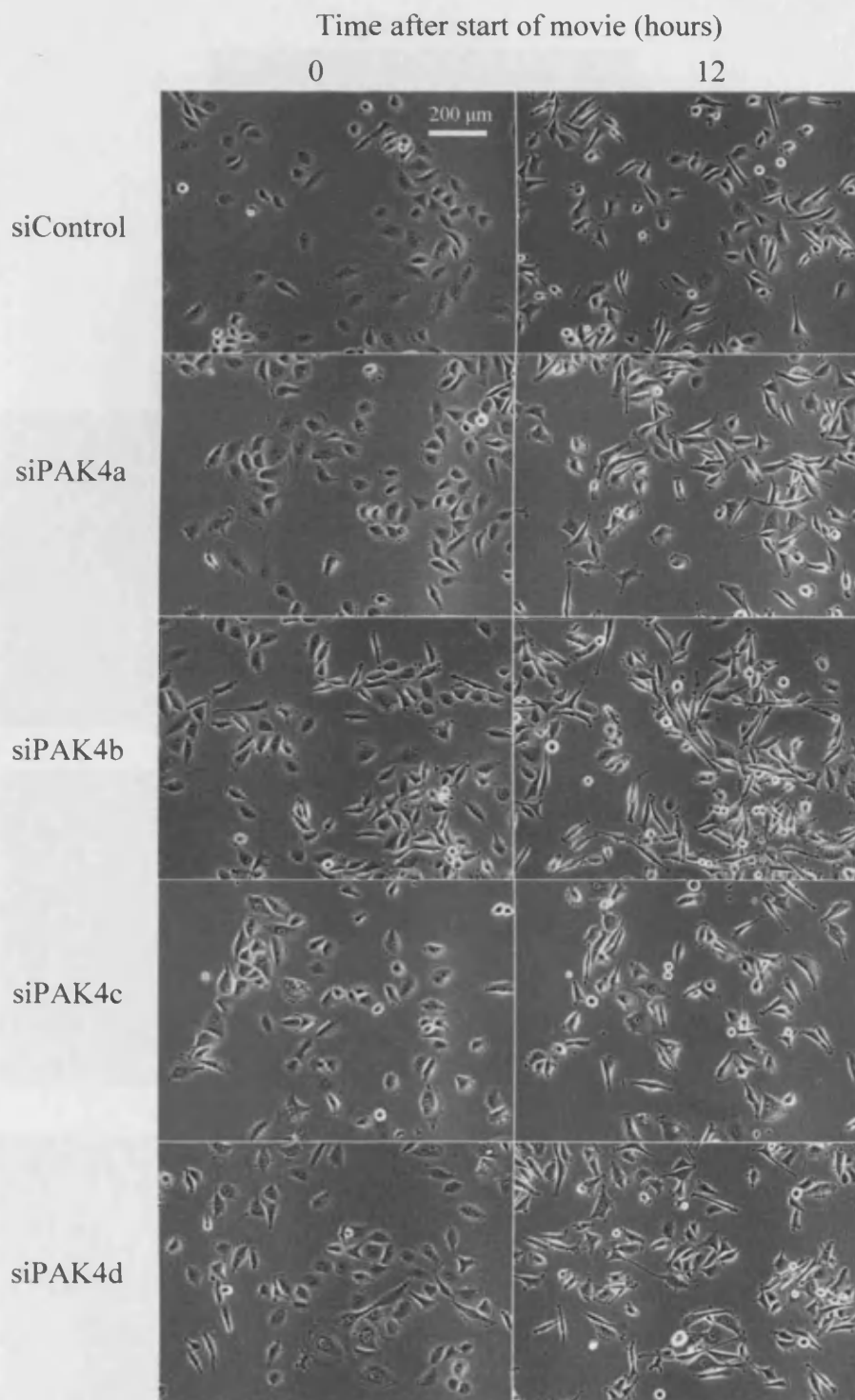


Figure 4.18: PAK4 knockdown does not detectably affect PC3 morphology. PC3 cells were seeded and allowed to grow for 18 hours before transfection with control siRNA or one of four different oligomers against PAK4. Forty-eight hours after transfection, cells were stimulated with 10 ng/ml HGF and movies were made for 16 hours taking one frame every 10 minutes (0 and 12 hour timepoints shown). Movies are representative of two repeat experiments.

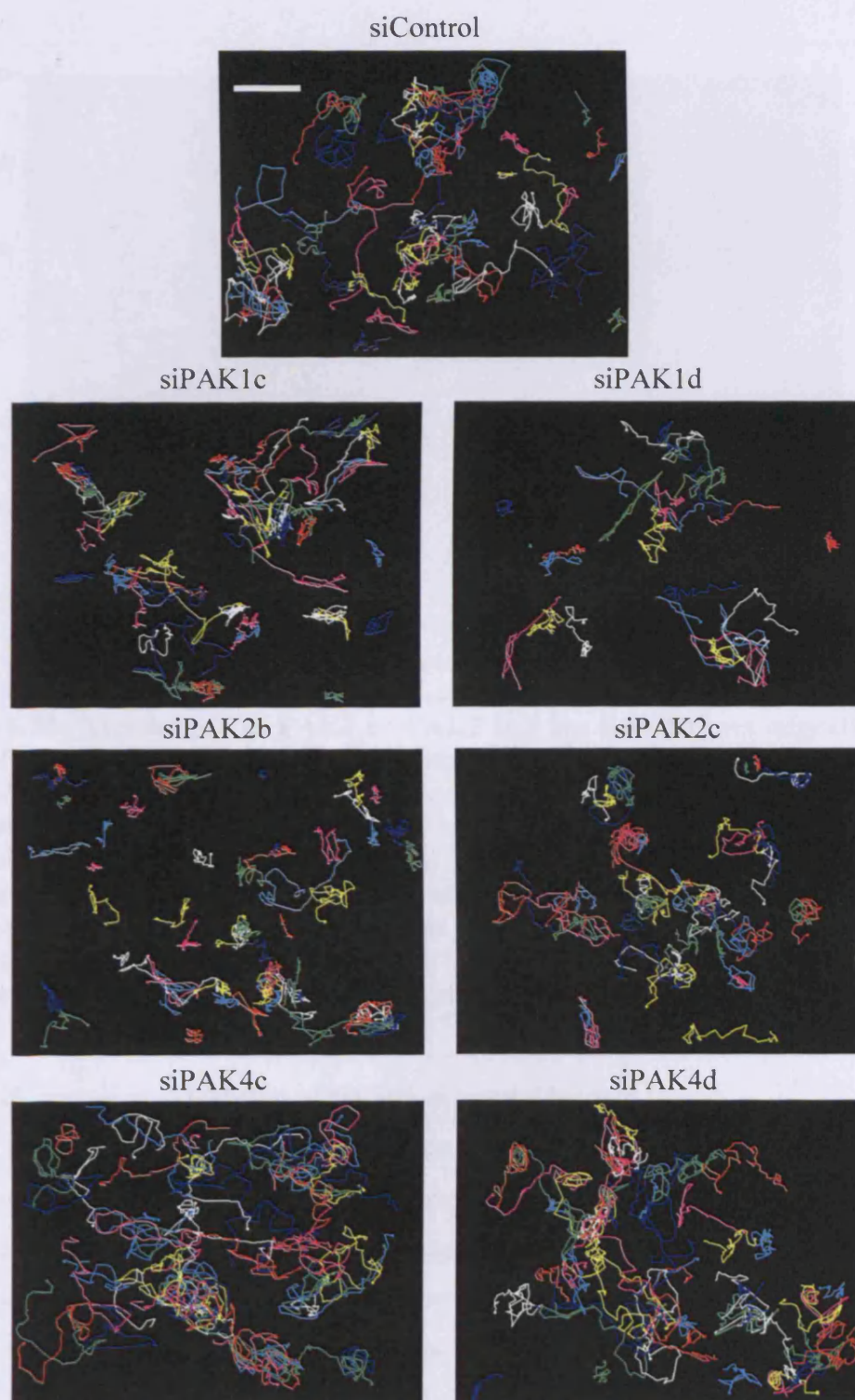


Figure 4.19: PAK2 affects persistence of PC3 cell migration.

PC3 cells were treated with control siRNA or siRNA against PAK1, PAK2 or PAK4. 48 hours after transfection movies were made for 12 hours taking one frame every 10 minutes. Cells which did not divide, leave the field of view or become obscured by other cells were tracked. Tracks from one movie are shown, dots denote the finishing position of cells at 12 hours after HGF stimulation.

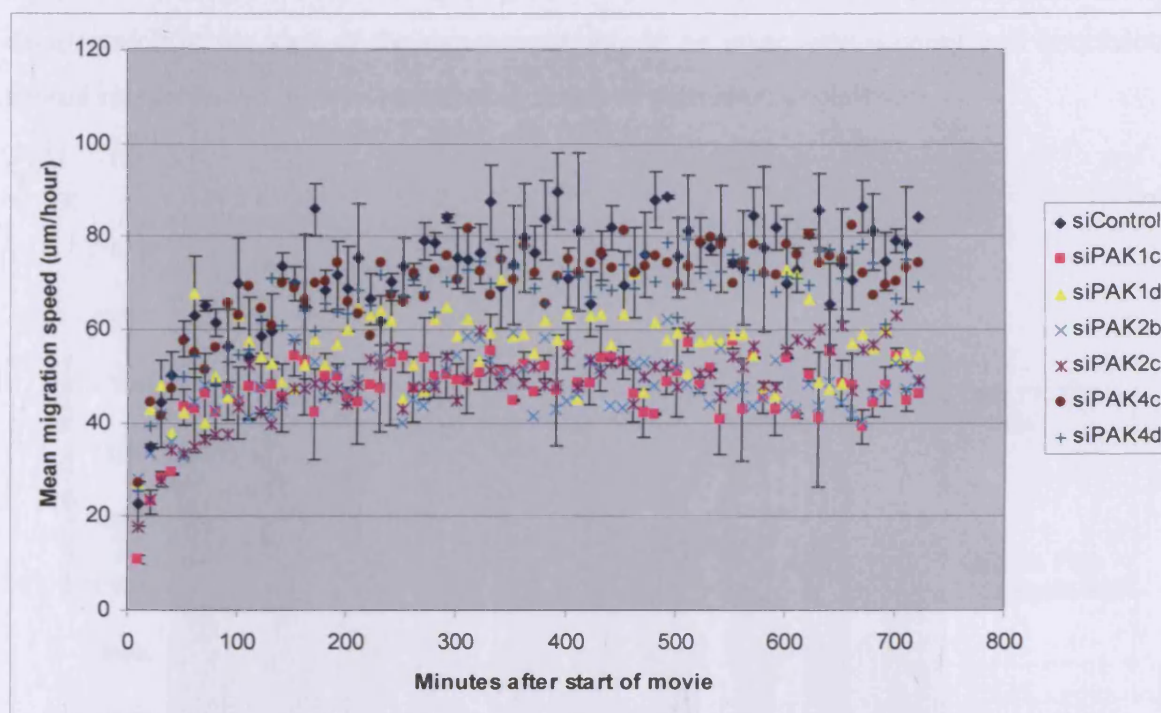


Figure 4.20: Knockdown of PAK1 or PAK2 but not PAK4 slows migration speed of PC3 cells. PC3 cells were treated with control siRNA or siRNA against PAK1, PAK2 or PAK4. Forty-eight hours after transfection movies were made for 12 hours taking one frame every 10 minutes. Cells which did not divide, leave the field of view or become obscured by other cells were tracked and their mean speed at each time point was calculated. Data was taken from two independent experiments. Error bars for siControl and siPAK1c are shown and represent the means of two experiments. Data are from at least 120 cells over two experiments for PAK1 and PAK2 knockdown. Data for PAK4 knockdown is from one experiment only tracking at least 140 cells.

4.7: HGF increases migration of DU145 in wound healing assays

Wound healing assays have previously been used to assess collective cell migration and a high throughput, automated assay of wound healing has been described (Yarrow *et al.*, 2005). To investigate whether a high-throughput assay could be used to generate multiple experimental repeats of wound healing, and so be used to screen for the effects of PAK knockdown, a similar approach to Yarrow *et al.* was used. Confluent monolayers of DU145 cells were scratched manually and allowed to heal for 16 hours before automatic imaging and calculation of scratch area. Due to variation in the size of the scratches at the start of the experiment, this assay was not sensitive enough to measure small changes which might be present between conditions. However, the size of the scratches after 16 hours was smaller in the presence of 10 ng/ml HGF showing that HGF increases the speed

of wound healing in DU145 cells (Figure 4.20). To improve this experiment, the area of each scratch at the start of the experiment should be taken into account and knockdown should be confirmed by western blotting for each individual experiment.

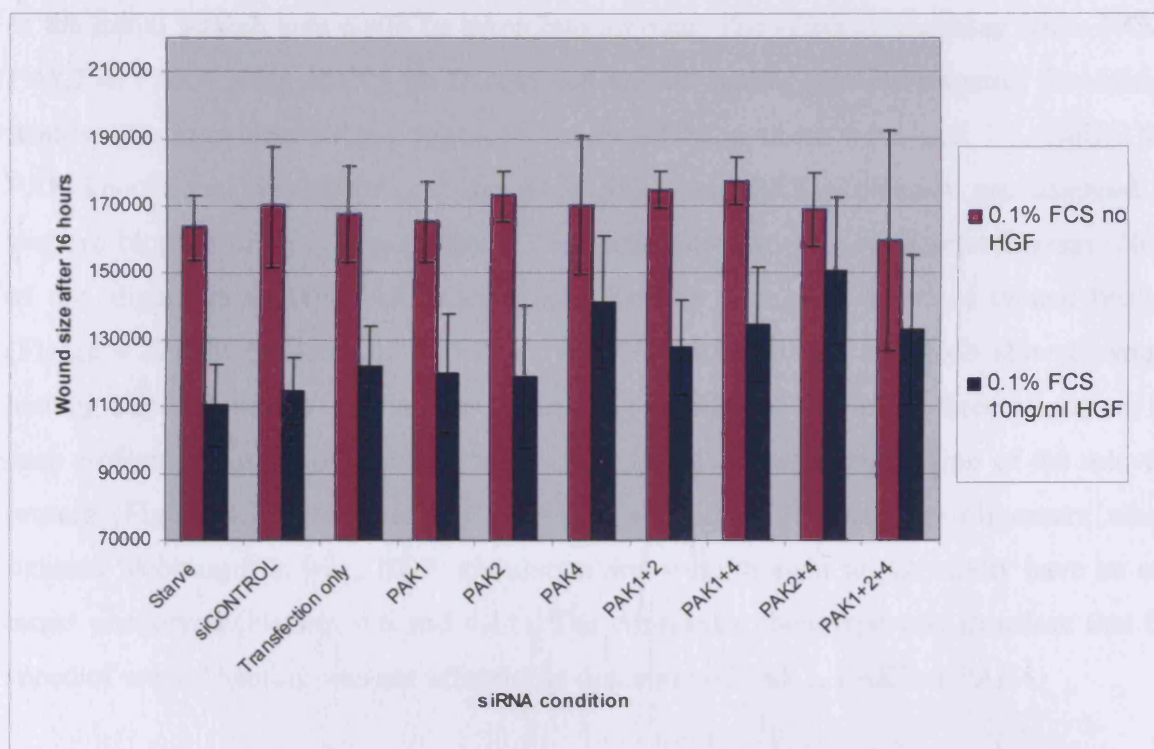
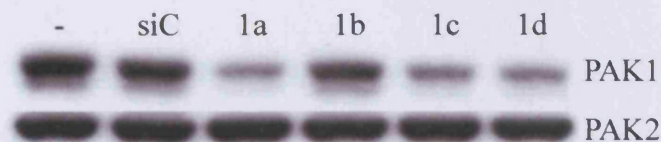
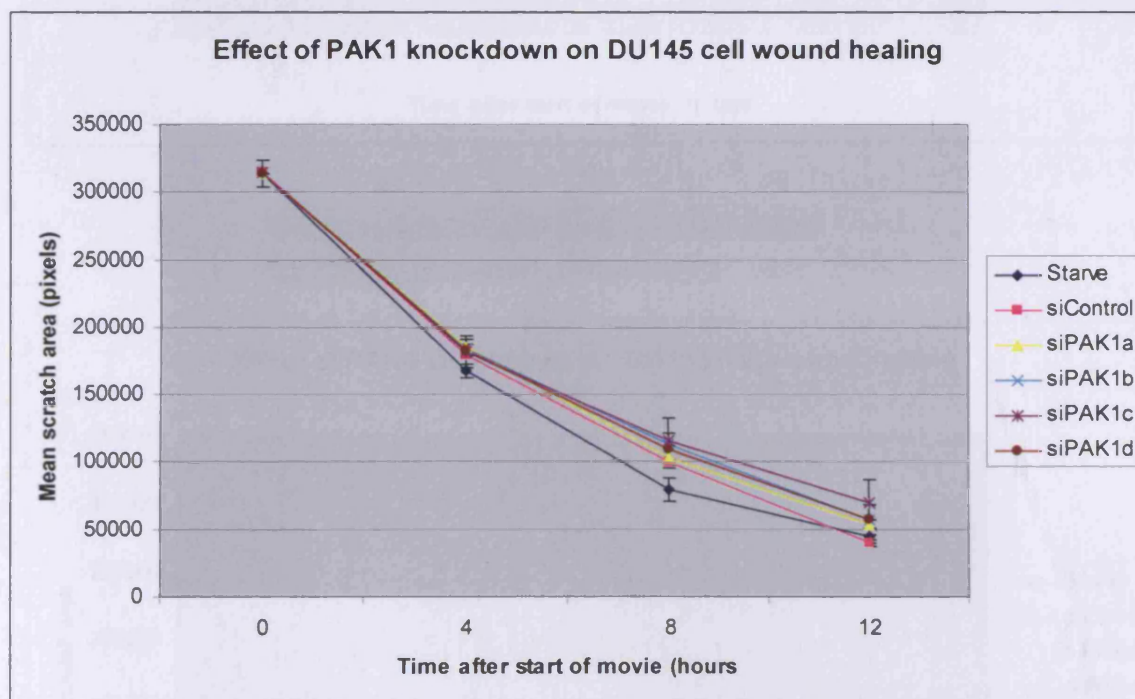


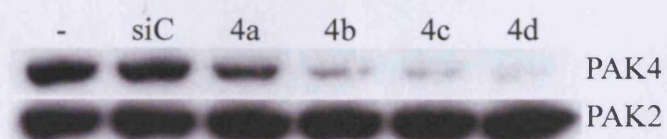
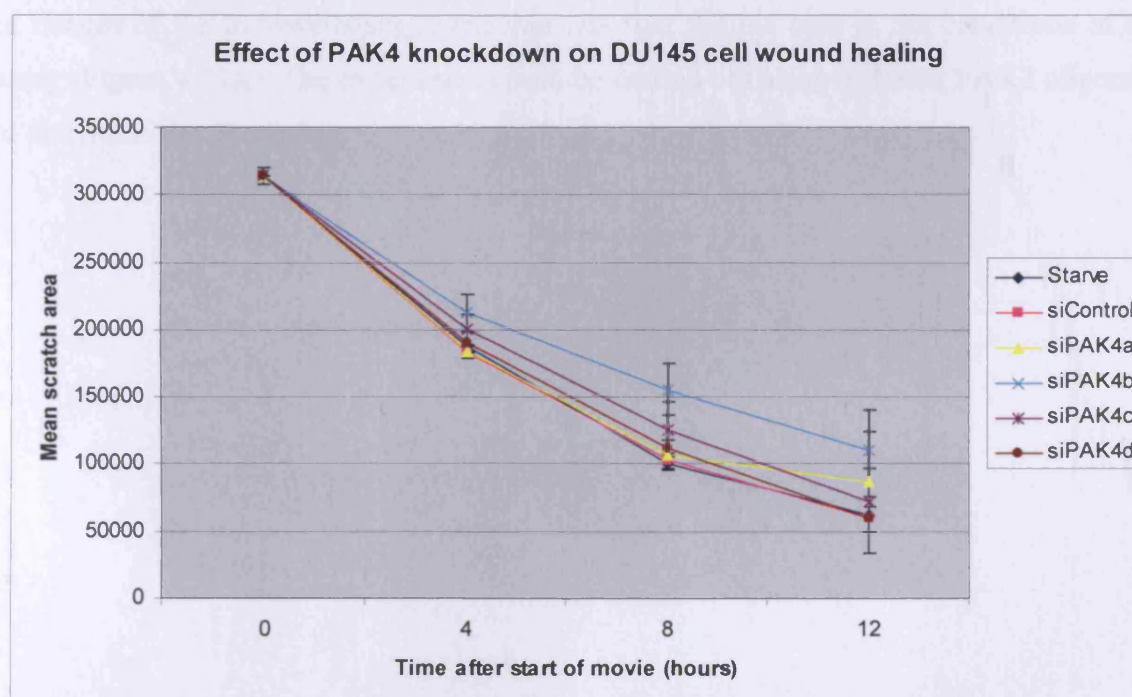
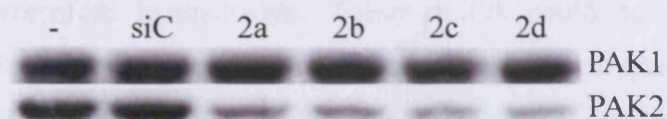
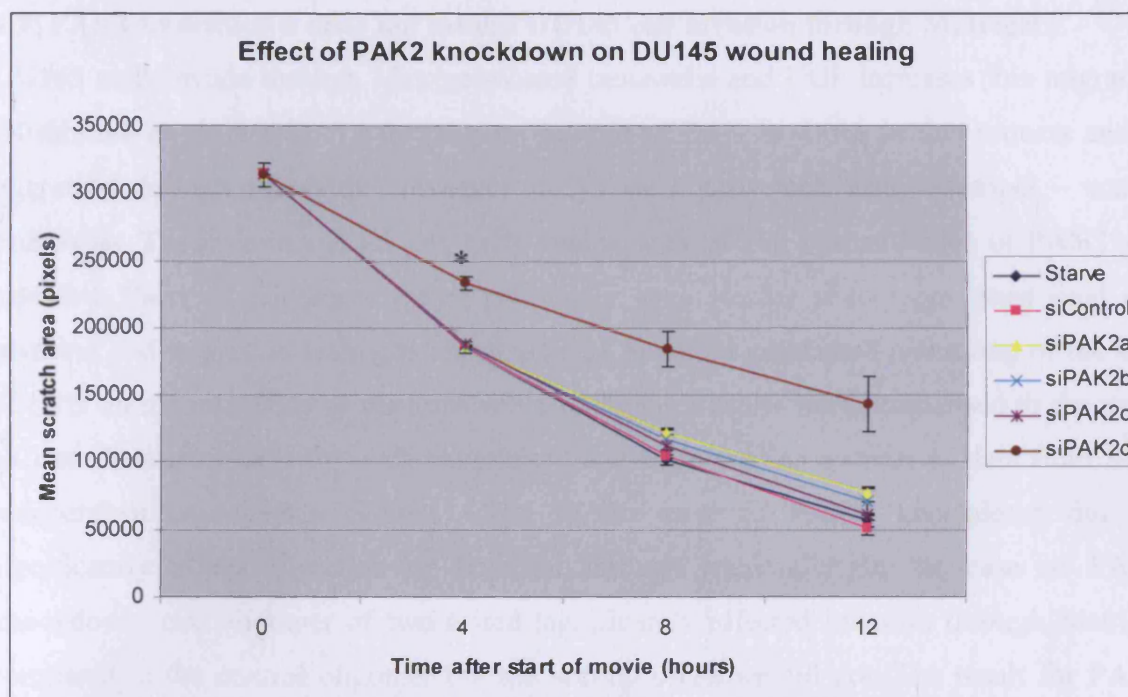
Figure 4.21: HGF increases speed of wound healing in DU145 cells. DU145 cells were seeded in 96-well plates and allowed to grow for 18 hours prior to transfection with control siRNA or single oligomers of PAK siRNA. PAK oligomers used were PAK1a, PAK2b and PAK4b. The control oligomer was from Qiagen. Forty-eight hours after transfection when cells had reached confluence, the cells were starved in 0.1% FCS for 8 hours then scratched with a 20 μ l pipette tip. Medium was changed for fresh starvation medium or starvation medium supplemented with 10 ng/ml HGF and scratches were allowed to heal for 16 hours. The cells were then fixed in 3.8% formaldehyde, stained with rhodamine-phalloidin and Hoescht, and imaged automatically using a Cellomics ArrayScan imaging system taking an image of the centre of each well. The area of the scratch in the resulting images was measured using the Definiens program. Data from wells where the scratch was not in the centre of the well were not included in the analysis leaving $11 \geq n \geq 4$. The graphs include data from one experiment and as such, significance can not be measured. Error bars represent standard deviation from the mean of results from individual wells.

4.8: PAK knockdown does not slow DU145 cell wound healing

The semi-automated wound healing assay (Figure 4.21) could not reliably measure small changes in wound healing that might be caused by PAK knockdown. To overcome this, a manual approach was taken in which movies were made of wound healing so that variation of the initial scratch area could be taken into account. The effect of knocking down PAK1, PAK2 or PAK4 using siRNA on DU145 cell wound healing was investigated. To validate results, four individual siRNA oligomers for each PAK isoform were used. To confirm that PAK knockdown was effective under these conditions, PAK expression was assessed by western blotting using lysates prepared from cells after carrying out a scratch assay. None of the oligomers against PAK1 caused a significant change in speed of wound healing (Figure 4.22). In the case of PAK2 and PAK4, siPAK2d and siPAK4b slowed wound healing although only one point was statistically significant. The other three oligomers for each isoform did not slow healing, although they all decreased expression of the relevant protein (Figure 4.22). Interestingly siPAK2d and siPAK4b were the oligomers which induced blebbing following HGF stimulation and were thought to potentially have an off-target phenotype (Figures 4.6 and 4.11). The consensus phenotype was therefore that the speed of wound healing was not affected by depletion of PAK1, PAK2 or PAK4.

Figure 4.22: PAK knockdown does not affect the speed of DU145 cell wound healing. Graphs showing wound area during wound healing in the presence of HGF. Movies of wound healing assays were made using DU145 cells treated with siRNA against PAK1, PAK2 or PAK4 and the area of the scratch was measured at 4 hourly intervals after the start. In each movie, results were normalised to the largest starting scratch in the dataset. Error bars represent standard error of the mean. Each experiment was carried out in quadruplicate and graphs incorporate data from three (PAK1) or two (PAK2 and PAK4) repeat experiments. Error bars represent standard error of the mean. * = $p \leq 0.05$ two tailed T test. Western blots were performed using lysate taken from DU145 cells after performing a scratch assay. Blots were probed with antibodies against PAK1, PAK2 or PAK4 as indicated. siRNA treatment is indicated, siC = control siRNA (Dharmacon), - = untransfected control.

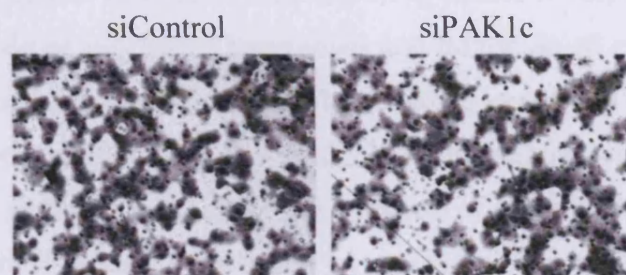




4.9: PAK1 knockdown does not inhibit DU145 cell invasion through Matrigel

DU145 cells invade through Matrigel-coated transwells and HGF increases this migration (Nishimura et al., 1998). To investigate the role of PAK isoforms in this process and in migration through transwells, invasion assays were performed using Matrigel – coated transwells. The invasion of DU145 cells treated with siRNA against PAK1 or PAK2 was assessed. Pairs of oligomers which previously gave similar phenotypes were used and invasion and migration were quantified after 24 hours by automated measuring of the area of cells on the underside of the transwell membrane. Results were normalised to the mean siControl measurement for each experiment and expressed as a mean of data from three independent experiments (Figure 4.23). In the case of PAK1, knockdown did not significantly affect migration or invasion through transwells. In the case of PAK2 knockdown, one oligomer of two tested significantly affected invasion through Matrigel compared to the control oligomer but the second oligomer did not. The result for PAK2 knockdown was therefore inconclusive. These results could be checked by directly counting the number of cells which had transmigrated rather than quantifying area. In time lapse movies PAK1 knockdown affected rounding, and so cell area, but visual observation of images of the transwells suggested that this was not the case in the conditions of this assay (Figure 4.23A). The experiment could be carried out using different PAK2 oligomers to determine the phenotype.

A



B

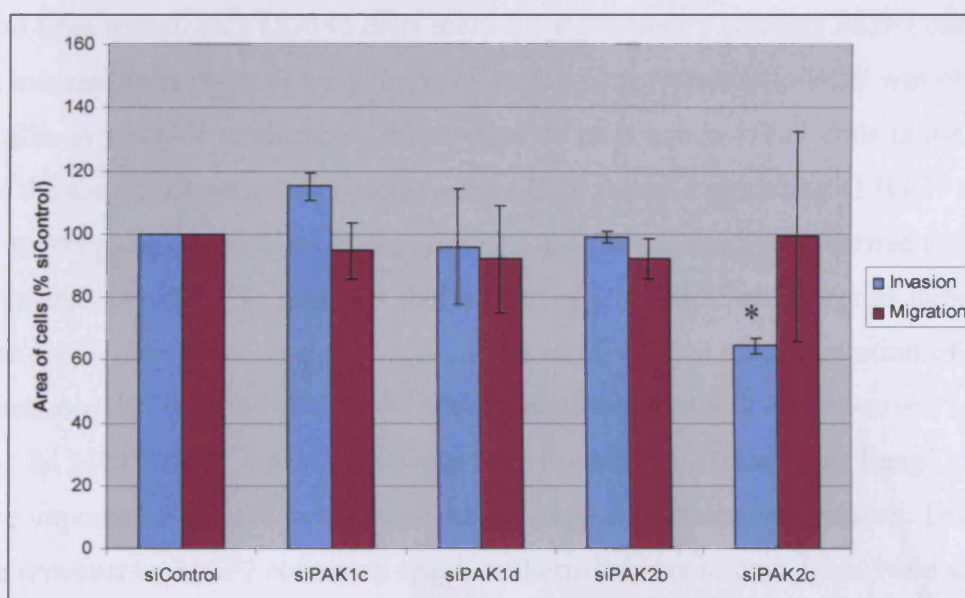


Figure 4.23: Knockdown of PAK1 in DU145 cells does not affect invasion through Matrigel or migration through transwells. DU145 cells were transfected with control siRNA or one of two different oligomers against PAK1 or PAK2. After 48 hours, 10^5 of the pre-treated cells were seeded in Matrigel-coated transwells and 2×10^4 were seeded in uncoated transwells, each with 40 ng/ml HGF in the lower chamber as a chemoattractant. After a further 24 hours, cells on the bottom of the transwells were fixed and stained with crystal violet and images of five different fields of view were acquired using a Nikon TL microscope (A). The amount of migration or invasion was quantified by measuring the mean stained area over the five fields of view (B). Graphs represent data from three repeat experiments for siControl, siPAK1c, siPAK1d and siPAK2b, and two experimental repeats for siPAK2c. Error bars represent standard error of the mean. * = $p \leq 0.05$, two tailed student's t test.

4.10: Discussion

Recently, DU145 cancer cells have been shown to scatter in response to HGF providing a model for the study of junctional breakdown and migration of cancer cells (Miura et al., 2001). HT29 colon cancer cells have also been shown to respond to HGF by increased cell spreading, but not scattering (Fazekas et al., 2000; Nabeshima et al., 1998) and this could also be used as a different readout for the response of cancer cells to HGF. In the present study DU145 and HT29 cells were tested as well as MCF7 and PC3 cells to set up assays for the investigation of the role of PAKs in cancer cell migration and scattering. Of the three cell lines tested, only DU145 cells scattered significantly whereas MCF7 only showed a slight increase in ruffling at early time points. No clear response to HGF was observed in HT29 cells. A possible explanation for the lack of response in HT29 cells is the substrate used for the assay; previous experiments where HGF induced spreading of HT29 cells were carried out on glass (Nabeshima et al., 1998) but the present study was carried out on tissue culture-treated plastic. This suggests that engagement of different integrins dependent on substrate could play a role in the spreading response. HGF at a concentration of 40 ng/ml was tested on HT29 cells in 0.1% FCS and scattering was still not observed (result not shown). In MCF7 cells, c-Met associates with E-cadherin (Hiscox and Jiang, 1999) so it might be expected that HGF stimulation would trigger junctional breakdown. This was not the case however so MCF7 cells may require other stimuli to scatter. It has been shown that overexpression of the cell adhesion molecule L1 disrupts junctions in MCF7 cells, and knockdown of endogenous L1 leads to more compact colonies (Shtutman et al., 2006). Thus, MCF7 scattering may depend on regulation of L1.

The effect of PAK1, PAK2 or PAK4 knockdown on random migration of PC3 cells and DU145 cell scattering or wound healing in response to HGF was investigated. For each isoform, variation in phenotype was observed between different oligomers. This variation is likely to occur as a result of off-target effects of the siRNA sequences. It has been shown using microarrays that transfection of siRNA into cells causes off-target knockdown of many unrelated mRNA transcripts (Chi et al., 2003). It was observed that many of these off-target mRNAs had sequence similarity between the mRNA and the 5' end of the antisense strand (the seed region) implying that the knockdown could work in the same way as the 5' end of microRNAs targeting the 3' untranslated region (UTR) of an mRNA (Jackson 2003). Indeed base substitution in the seed region was found to rescue off-target

knockdown of many mRNAs (Jackson et al., 2006). Further to this *Birmingham et al.* developed a web-based tool for the prediction of possible off-target genes (Birmingham et al., 2006). When the sequences for the PAK siRNAs used in this study were input into this tool, lists of between 60 and 2391 potential off-target genes with multiple seed-region matches were generated highlighting the need to control for possible off-target effects by using more than one siRNA oligomer in experiments. These predictions are not always correct, but they provide a possible means to suggest how an off-target effect could occur. For example, the seed region of siPAK1a has multiple matches to the mRNA for β -PIX (accession number NM_145735), a known PAK interactor, which plays a role in focal adhesion turnover. Interestingly, this oligomer did not give an identifiable phenotype with cells scattering following HGF stimulation in the same way as control cells. It would be interesting to know if this was due to off-target knockdown which rescued the effect of PAK1-depletion. Potential off-target effects could be investigated further by western blotting. Recently, modification of the 5' end of the antisense strand of siRNA has been shown to reduce off-target knockdown (Snove and Rossi, 2006). 'On target' oligomers with this modification are available (Dharmacon) and could be used in future studies

Because of the presence of off-target effects it would have been desirable to carry out more extensive controls such as re-expression of the target protein following siRNA knockdown. This was attempted in DU145 cells but lipid-mediated transfection of DNA plasmids encoding PAK isoforms into cells had a very low efficiency and adversely affected the cells causing changes in morphology and behaviour (result not shown). Therefore it was not possible to carry out this control experiment and the consensus response to two or three of the four oligomers was taken to be the most likely phenotype of knockdown. However, it should be kept in mind that a consensus response between oligomers could still be influenced or caused by off-target effects.

In DU145 cell scattering assays, depletion of PAK1 led to a phenotype where fewer cells detached completely from colonies and cells remained more spread than control cells. Confocal microscopy and actin staining showed that more cells retained their E-cadherin-based junctions than control cells. The observed phenotype agrees with previous research in keratinocytes showing that Rac1 induces junctional breakdown through PAK1 (Lozano et al., 2008) and suggests that this could be the case in HGF-induced scattering. Preliminary

Rac1 knockdown experiments showed that the phenotype they induce in DU145 cells is similar to that observed with PAK1 knockdown. The relationship between Rac1 and PAK1 in DU145 cells should be investigated further in future work to elucidate their roles in junctional breakdown downstream of HGF. The decreased cell rounding of PAK1-deficient DU145 cells compared to control cells following HGF stimulation could be due to an increase in adhesion through focal contacts. Overexpression of GIT1 and PAK-interacting exchange factor (PIX) lead to disassembly of focal adhesions and interact in complex with PAK (Zhao et al., 2000). It would be interesting to investigate whether PAK1 depletion affects focal adhesion breakdown through GIT1, or whether knockdown of GIT1 in DU145 cells would have a similar effect to PAK1 knockdown on spreading. However, it has previously been shown that knockdown of GIT1 in HeLa cells inhibits EGF-induced cell spreading (Yin et al., 2005). Increased adhesion could also contribute to the decrease in migration seen in PAK1-depleted PC3 cells. However, the migration of DU145 cells in wound healing assays was unaffected.

Another mechanism that could lead to increased spreading is a reduction in contractility. This could be through the ability of PAK1 and PAK2 to phosphorylate regulatory myosin light chain (R-MLC) at serine 19 leading to increased contraction (Kiosses et al., 1999; Zeng et al., 2000). This is backed up by evidence showing that active PAK1 increases permeability of vascular endothelial cells, and this corresponds to phosphorylation of MLC (Stockton et al., 2004). However, PAK1 and PAK2 can also lead to decreased contractility by phosphorylation and inactivation of myosin light chain kinase (Goeckeler et al., 2000; Sanders et al., 1999). The dual role of PAK1 and PAK2 in regulation of R-MLC contractility suggests that their activity towards either R-MLC or MLCK depends on the cell context and the activity of other signalling pathways.

DU145 cells depleted in PAK2 were able to scatter but cells had broader lamellipodia, particularly when using oligomer siPAK2b, although results need to be repeated to determine their significance. This was further observed in confocal images of DU145 cells stained for actin filaments. Constitutively active Rac1 has been shown to induce the formation of broader lamellipodia in PtK1 cells and this effect is blocked by expression of dominant negative LIMK1 or the PAK1 AID (Delorme et al., 2007). In chapter 1 it was found that PAK2 knockdown leads to an up-regulation of phosphorylation of PAK1 at

S199/204. Phosphorylation of this site reduces β -PIX binding (Mott et al., 2005) and so could lead to a redistribution of PAK from focal contacts to the cytoplasm. To investigate whether PAK1 phosphorylation plays a role in the PAK2 knockdown phenotype, PAK1 and PAK2 were knocked down simultaneously. The combination of PAK2b and PAK1d oligomers did alter the lamellipodia making them flatter and more like the PAK1b phenotype. Yet use of PAK1c and PAK2c together did not alter the lamellipodia compared to PAK2c alone. Therefore, in these conditions, PAK1 knockdown did not rescue the PAK2-knockdown phenotype but knockdown of two proteins simultaneously using two different pairs of oligomers does not give identical results even when each of the pairs of oligomers alone give a similar phenotype. This could be due to the combination of two oligomers leading to a combination of off target knockdown which gives a different cell phenotype highlighting the importance of validating results with more than one separate siRNA oligomer.

It has also been shown that PAK1, PAK2 and PAK4 can activate LIMK leading to cofilin phosphorylation and inactivation (Misra et al., 2005) and dephosphorylated cofilin is required for lamellipodia extension (Kiuchi et al., 2007). This suggests that PAK knockdown could lead to an increase in active cofilin which could be responsible for the morphology of the lamellipodia observed in PAK2-deficient cells. This appears contrary to the finding that dominant negative LIMK1 blocks the Rac1-induced formation of broad lamellipodia (Delorme et al., 2007), but lamellipodium extension also depends on a free pool of actin monomers and free barbed ends in the leading edge, both supplied by cofilin.

PAK1 or PAK2 knockdown slowed the speed of PC3 migration in the presence of HGF. As well as a possible role for adhesion in impaired PC3 migration speed, cell polarity may have an effect. PAK is thought to play multiple roles in cell polarisation and maintenance of polarity. PAK1 can phosphorylate Op18/Stathmin so promoting microtubule stability (Wittmann et al., 2004). Active PAK1 is localized at the leading edge of cells and is required for restricting Rac-dependent actin polymerisation to that location (Cau and Hall, 2005). In this way, PAK1 would promote leading edge protrusion and loss of PAK1 could lead to a defect in cell polarisation.

In the case of knockdown of PAK4 each of the oligomers gave a slightly different phenotype so it was unclear which was the most likely phenotype of PAK4 knockdown. In a recent study, PAK4 knockdown in PC3 cells led to cell rounding following HGF stimulation (Ahmed et al., 2008). This was not observed in the present study with any of the oligomers, possibly due to the conditions used as the cells were starved in the *Ahmed* study, but it could also be due to oligomer-specific effects. In this study, siPAK4b led to a phenotype where DU145 cells blebbed more following HGF stimulation. This is similar to the phenotype seen with Cdc42 knockdown in DU145 cells and Cdc42 has been shown to activate PAK4 in response to HGF in MDCK cells (Wells et al., 2002). However, as only one oligomer gave this phenotype it is more likely to be an off-target effect. Oligomer siPAK4b also gave an anomalous effect in PC3 cells where cells became more elongated than control cells following HGF stimulation and in DU145 cells where wound healing was inhibited. The oligomers siPAK4c and siPAK4d had no significant effect on PC3 morphology or speed of migration suggesting that PAK4 is not important for PC3 migration. They also had no significant effect on the speed of DU145 wound healing. Taken together, these results are not conclusive and show that further work is required to uncover the roles of PAK4 in DU145 and PC3 responses to HGF. A possible approach would be to use 'on-target' siRNA oligomers with a 5' modification to decrease off-target effects.

The effect of PAK knockdown on invasion of DU145 cells through Matrigel and migration through transwells was also investigated. PAK1 knockdown did not affect invasion or migration in this assay. This is contrary to previous work in melanocytes where knockdown of PAK1 blocked invasion through Matrigel, although only one oligomer was used so an off-target effect cannot be ruled out (Pavey et al., 2006). In the case of PAK2 knockdown, only one oligomer of two tested significantly inhibited invasion so this result was inconclusive.

Taken together, these results show that PAK1, PAK2 and PAK4 play distinct roles in the scattering of DU145 cells and migration of PC3 cells in response to HGF. The key phenotypes observed in PAK-depleted cells are summarised (table 4.1). PAK1 knockdown impaired PC3 migration and led to more spread DU145 cells. PAK2 knockdown also impaired PC3 migration and led to larger lamellipodia with different actin staining in

DU145 cells. The likely role of PAK4 in DU145 and PC3 cells was unclear as the four oligomers gave different phenotypes in DU145 cell scattering. Moreover, the two PAK4 oligomers tested in PC3 cells had no effect on cell migration speed.

PAK isoform	DU145 cell wound healing	DU145 scattering	PC3 cell migration	DU145 cell invasion
PAK1 knockdown	Unaffected	Scattering decreased	Migration speed decreased	Unaffected
PAK2 knockdown	Unaffected	Scattering not inhibited, broad lamellipodia	Migration speed decreased	No consensus phenotype
PAK4 knockdown	Unaffected	No consensus phenotype	Unaffected	Not tested

Table 4.1: Observed effects of PAK isoform knockdown in DU145 and PC3 cells.

Chapter 5: The role of PAK in HGF-induced cell signalling**5.1: Introduction**

In Chapter 4 it was shown that PAK1 and PAK2 play distinct roles in cancer cell responses to HGF. PAK1 knockdown led to a decrease in HGF-induced scattering and cell rounding in DU145 cells, and slower migration of PC3 cells. PAK2 knockdown led to broader lamellipodia in DU145 cells and slower migration in PC3 cells.

Many binding partners and substrates of PAKs have been described, and it is thought that these are responsible for most of the phenotypes observed upon expression of constitutively active and dominant negative PAK. PAK-dependent control of lamellipodia formation is thought to be mediated by activation of LIM kinase 1 (LIMK1) (Edwards et al., 1999), which phosphorylates and inactivates cofilin, blocking its actin severing and depolymerisation functions (Arber et al., 1998; Yang et al., 1998). PAK1, PAK2 and PAK4 have each been shown to activate LIMK1 (Dan et al., 2001a; Edwards et al., 1999; Misra et al., 2005) providing a pathway through which PAK could increase actin filament stability and block actin depolymerisation. As well as LIMK1, regulation of LIMK2 by PAK1, PAK2 and PAK4 in vitro has been suggested but results have not yet been published (Bokoch, 2003). It is thought that PAK can also regulate actin polymerisation and membrane ruffle formation through filamin A; an actin binding protein that induces cross-linking of F-actin into bundles (Vadlamudi et al., 2002).

PAK1 and PAK2 also regulate cell contractility. Both PAK1 and PAK2 have been shown to phosphorylate regulatory myosin light chain (R-MLC) directly leading to an increase in contractility (Chew et al., 1998; Ramos et al., 1997), but on the other hand can phosphorylate and inactivate myosin light chain kinase (MLCK) leading to a decrease in phospho-R-MLC (Goeckeler et al., 2000; Sanders et al., 1999).

The role of PAK in adhesion is believed to be mediated through its localisation to focal adhesions in complex with β -PIX and GIT. PAK1 binds directly to β -PIX and both proteins are localised to focal adhesions in complex with GIT1 via paxillin binding, it is thought that the localisation of these proteins is involved in focal adhesion turnover and lamellipodia formation (See Chapter 1, section 1.7) (Brown et al., 2002; Zhao et al., 2000).

PAKs can play a role in cell polarity through microtubule stability. In motile cells, microtubules extend towards the leading edge and it is thought that they play an important role in cell polarity (reviewed in Wittmann and Waterman-Storer, 2001). Microtubules are regulated through Op18/Stathmin which binds tubulin and decreases microtubule stability (Howell et al., 1999; Marklund et al., 1996). Op18 can be phosphorylated and inhibited by PAK1 which promotes microtubule stability (Wittmann et al., 2004). In this way PAK1 activation at the leading edge of cells could facilitate the maintenance of cell polarity and persistence of migration.

PAKs also activate MAPK signalling downstream of some growth factors. This was originally indicated by the yeast PAK homologue Ste20, which is part of the yeast mating factor MAPK cascade (reviewed in Dan et al., 2001b). It has been suggested that mammalian Group 1 PAKs can influence signalling through the Raf/MEK/ERK pathway or the p38-MAPK pathway (Bagrodia et al., 1995; Eblen et al., 2002; Slack-Davis et al., 2003; Zhang et al., 1995). However, MAPK activation downstream of PAK appears to be cell type and stimulus dependent, as there are multiple pathways that can feed into MAPK signalling.

It is unclear what the isoform-specific pathways leading to a functional difference between PAK1 and PAK2 could be. RNAi provides an effective way to investigate this.

The aim of this chapter is to investigate the effect of the knockdown of PAK1 or PAK2 on protein signalling following HGF stimulation of cancer cells.

Results

5.2: PAK1 knockdown does not affect E-cadherin expression in DU145 cells following HGF stimulation

HGF stimulation of DU145 cells leads to cell scattering with a loss of E-cadherin from junctions and a decrease in the total level of E-cadherin (Miura et al., 2001). In keratinocytes, PAK1 is required for loss of E-cadherin from junctions downstream of active Rac1 (Lozano et al., 2008). Knockdown of PAK1 in DU145 cells caused cells to remain more spread and maintain more cell junctions after HGF treatment than control cells (Chapter 4, Section 4.3). To investigate whether knockdown of PAK1 in DU145 cells caused a defect in the down-regulation of E-cadherin, the total level of E-cadherin was determined in PAK1-depleted cells before and after HGF stimulation for 24 hours (Figure 5.1). In this preliminary experiment, down regulation of E-cadherin following HGF stimulation was observed in control cells as expected and knockdown of PAK1 appeared to increase the total level of E-cadherin but further experiments are required to confirm this. E-cadherin down-regulation following HGF stimulation was still observed in PAK1-depleted cells.

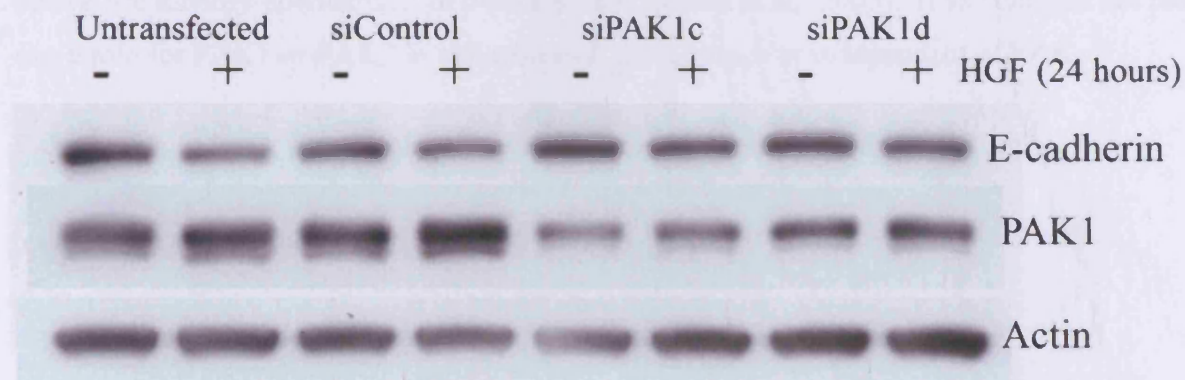


Figure 5.1: E-cadherin expression following HGF stimulation of control and PAK1-depleted DU145 cells. DU145 cells were seeded in growth medium for 18 hours prior to transfection with control siRNA or one of two oligomers of siRNA against PAK1. Cells were left untransfected as a further control. Forty-eight hours after transfection, cells were stimulated with 10ng/ml HGF in growth medium for 24 hours, or kept unstimulated in growth medium prior to lysis. Western blots were performed and probed with antibodies against E-cadherin, PAK1 or β -actin. Only one experiment was performed.

5.3 PAK1 or PAK2 knockdown does not affect phosphorylation of FAK downstream of HGF

In DU145 cells, PAK1-depleted cells remained more spread than control cells following HGF stimulation while PAK2-depleted cells had altered lamellipodia. In PC3 cells, knockdown of either PAK1 or PAK2 impaired cell motility (Chapter 4). These phenotypes could be partly due to increased adhesion. Focal adhesion kinase (FAK) is recruited transiently to lamellipodia at the leading edge of migrating cells where it is incorporated into new focal adhesions (Schlaepfer et al., 2004). FAK is known to be responsible for focal adhesion turnover and it has been shown that expression of dominant negative PAK1 blocks LPA-induced FAK phosphorylation in A2058 melanoma cells (Jung et al., 2004). This result suggests that in some situations, PAK1 could influence FAK tyrosine phosphorylation and activation. Phosphorylation of FAK at Y576/577 is required for full kinase activity (Ruest et al., 2000) so phosphorylation of these sites was investigated in PAK1 or PAK2-depleted cells (Figure 5.2 and 5.3). HGF stimulated the phosphorylation of FAK in DU145 and PC3 cells and knockdown of neither PAK1 or PAK2 knockdown affected this phosphorylation of FAK. This is probably because FAK is phosphorylated by active Src directly downstream of c-Met (Pongchairerk et al., 2005). These data do not rule out a role for PAK1 or PAK2 in cell adhesion downstream or independent of FAK.

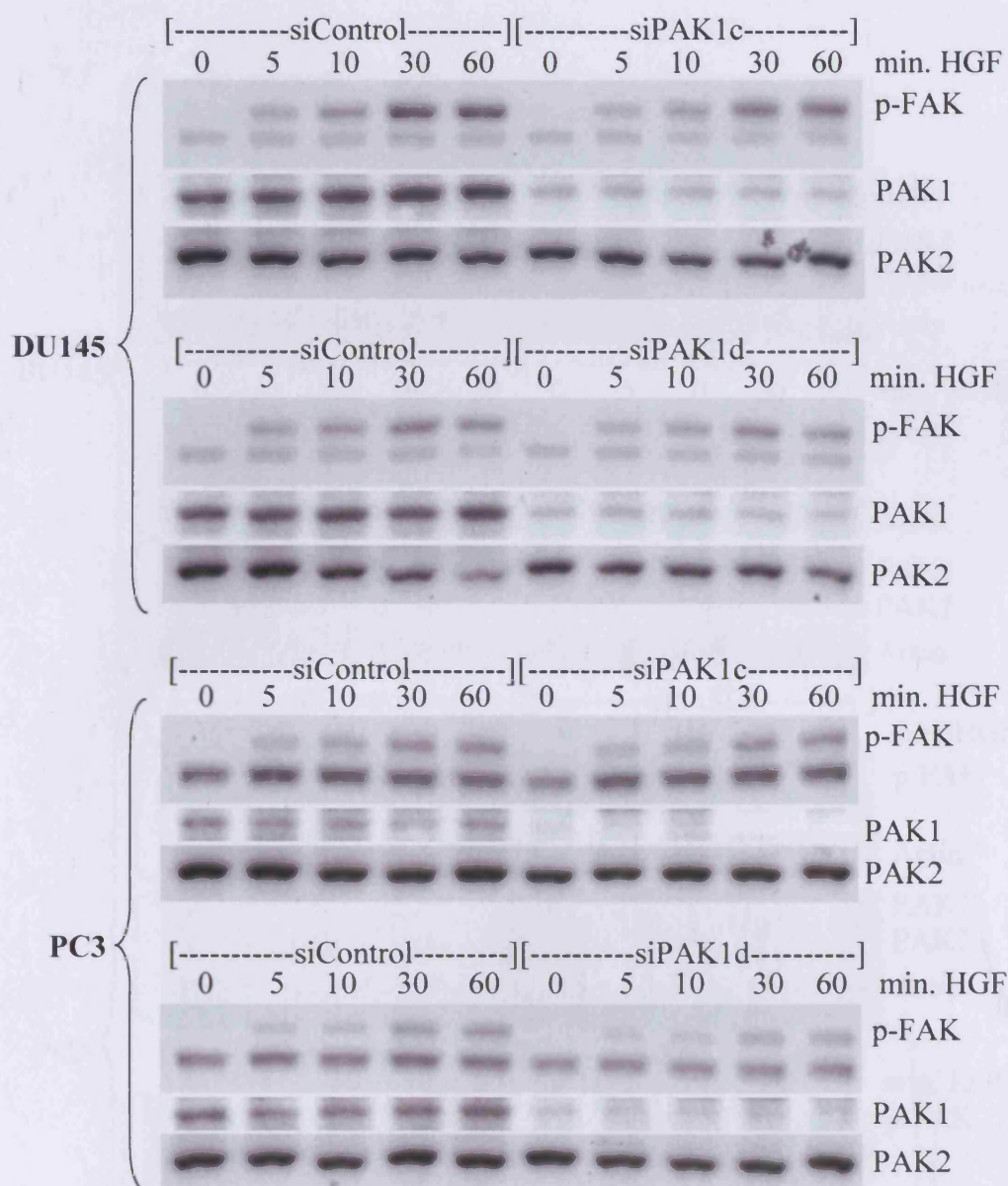


Figure 5.2: PAK1 knockdown does not affect phosphorylation of FAK following HGF stimulation in DU145 or PC3 cells. DU145 or PC3 cells were transfected with control siRNA or one of two siRNA oligomers against PAK1. Forty-eight hours after transfection, cells were stimulated with 10 ng/ml HGF for 0 – 60 minutes before lysates were prepared. Western blots were performed and probed with antibodies against phospho-FAK (Y576/577), PAK1 and PAK2. Some control blots are the same between Figures 3.10, 5.2, 5.17, 5.23 and 5.21. Blots are representative of two independent experiments.

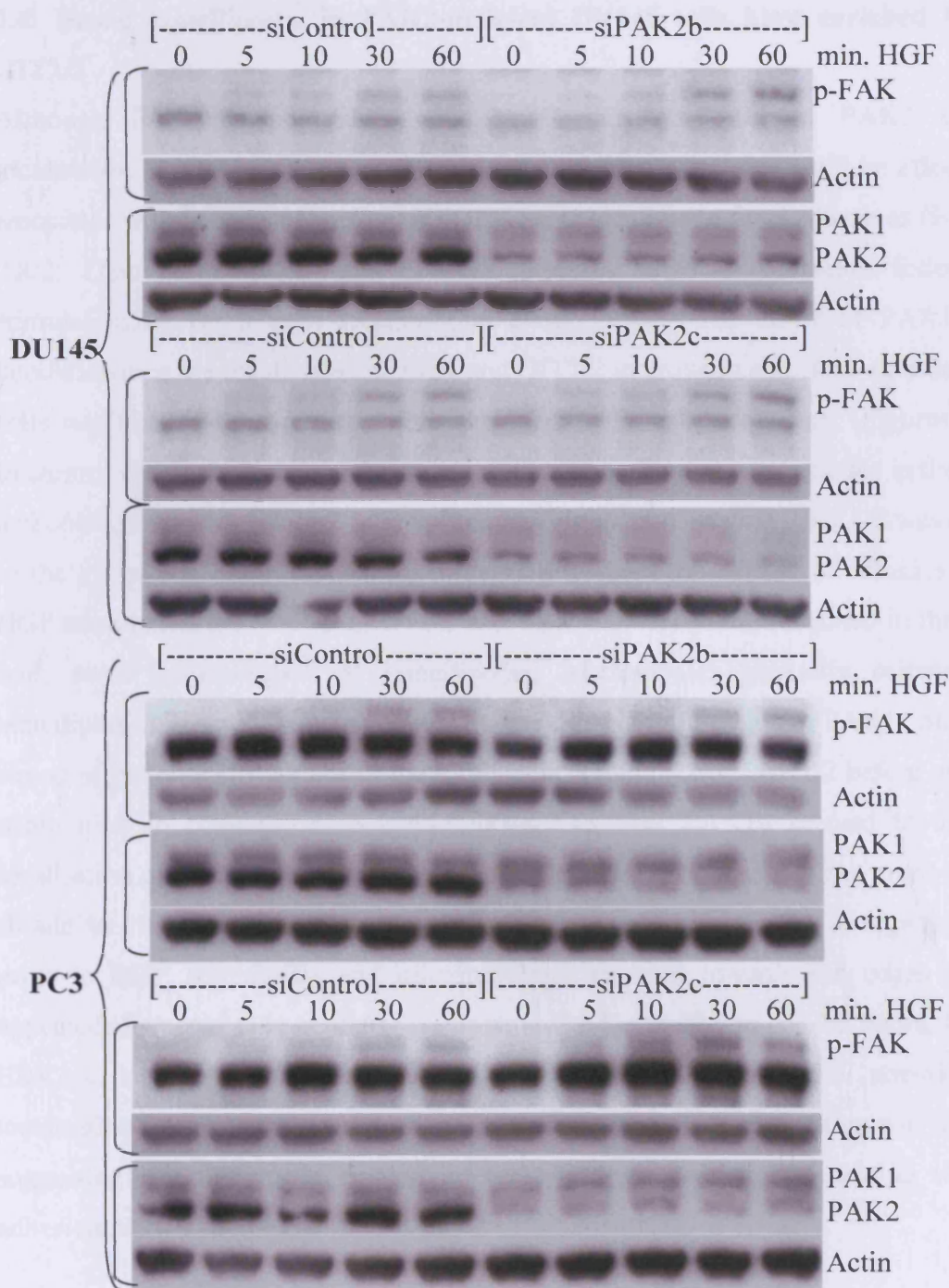


Figure 5.3: PAK2 knockdown does not affect phosphorylation of FAK following HGF stimulation in DU145 or PC3 cells. DU145 or PC3 cells were transected with control siRNA or one of two siRNA oligomers against PAK2. Forty-eight hours after transfection, cells were stimulated with 10 ng/ml HGF for 0 – 60 minutes before lysates were prepared. Western blots were performed and probed with antibodies against phospho-FAK (Y576/577) and β -actin or PAK1, PAK2 and β -actin. Straight left brackets indicate images from the same membrane. Some control blots are from the same membrane as in Figure 5.26 and 5.9. Blots are representative of two independent experiments.

5.4: Broad lamellipodia in PAK2-deficient DU145 cells have enriched ERK1 and GIT1/2

Although FAK phosphorylation was unaffected by PAK1 or PAK2 knockdown, localisation of proteins involved in focal adhesion dynamics could still be affected. PAK1 associates with β -PIX in complex with GIT1 and localises to focal adhesions (Brown et al., 2002; Zhao et al., 2000) and ERK1/2 localises to focal complexes following HGF stimulation of HeLa cells (Kermorgant et al., 2004). The effect of PAK1 or PAK2 knockdown on the localisation of ERK and GIT1/2 in growing and HGF-stimulated DU145 cells was observed by immunofluorescence and confocal microscopy. (Figures 5.4 – 5.7). In control cells prior to HGF stimulation, GIT1/2 localisation was punctate in the cytoplasm and colocalised with ERK1/2 in lamellipodia (Figure 5.4, arrows). ERK1/2 was also diffuse in the cytoplasm. Microtubules were oriented towards the outside of colonies. Following HGF stimulation for 18 hours, GIT1/2 and ERK1/2 localisation remained in the cytoplasm with some colocalisation at lamellipodia. Microtubules generally oriented towards lamellipodia (Figure 5.5 arrow). Knockdown of PAK1 with oligomer PAK1c did not have a strong effect on microtubules or the localisation of GIT1/2 or ERK1/2 before or after HGF stimulation in comparison to control cells. Oligomer PAK1d seemed to decrease the localisation of GIT1/2 and ERK1/2 in the cell periphery (Figure 5.5) but more experiments should be performed to confirm this. PAK2-depleted cells formed disorganised colonies prior to HGF stimulation and microtubule orientation towards the edges of colonies appeared disrupted (Figure 5.6). Following HGF stimulation for 18 hours, GIT1/2 and ERK1/2 localisation in lamellipodia was enhanced (Figure 5.7 arrows). ERK1/2 localisation in focal adhesion-type structures following HGF stimulation was not observed suggesting that this could depend on the cell type, however co-staining with a focal adhesion marker such as paxillin is required to show this.

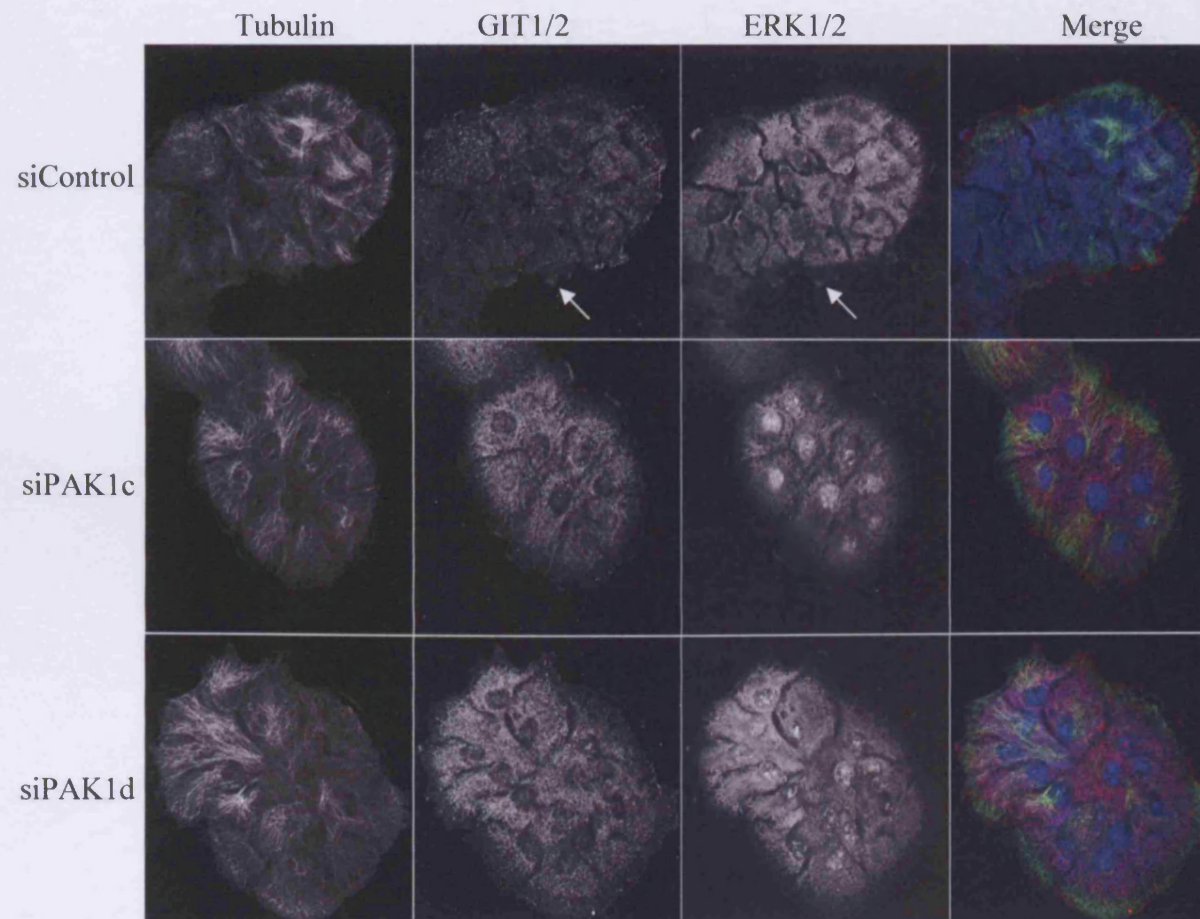


Figure 5.4: Effect of PAK1 knockdown on tubulin, GIT1/2 and ERK1/2 localisation in growing cells. DU145 cells were transfected with control siRNA or one of two siRNA oligomers against PAK1. 66 hours after transfection, cells were fixed in methanol and stained with antibodies against GIT1/2 and ERK1/2 followed by fluorescent secondary antibodies and a FITC-conjugated tubulin antibody. Arrows indicate colocalisation of ERK1/2 and GIT1/2.

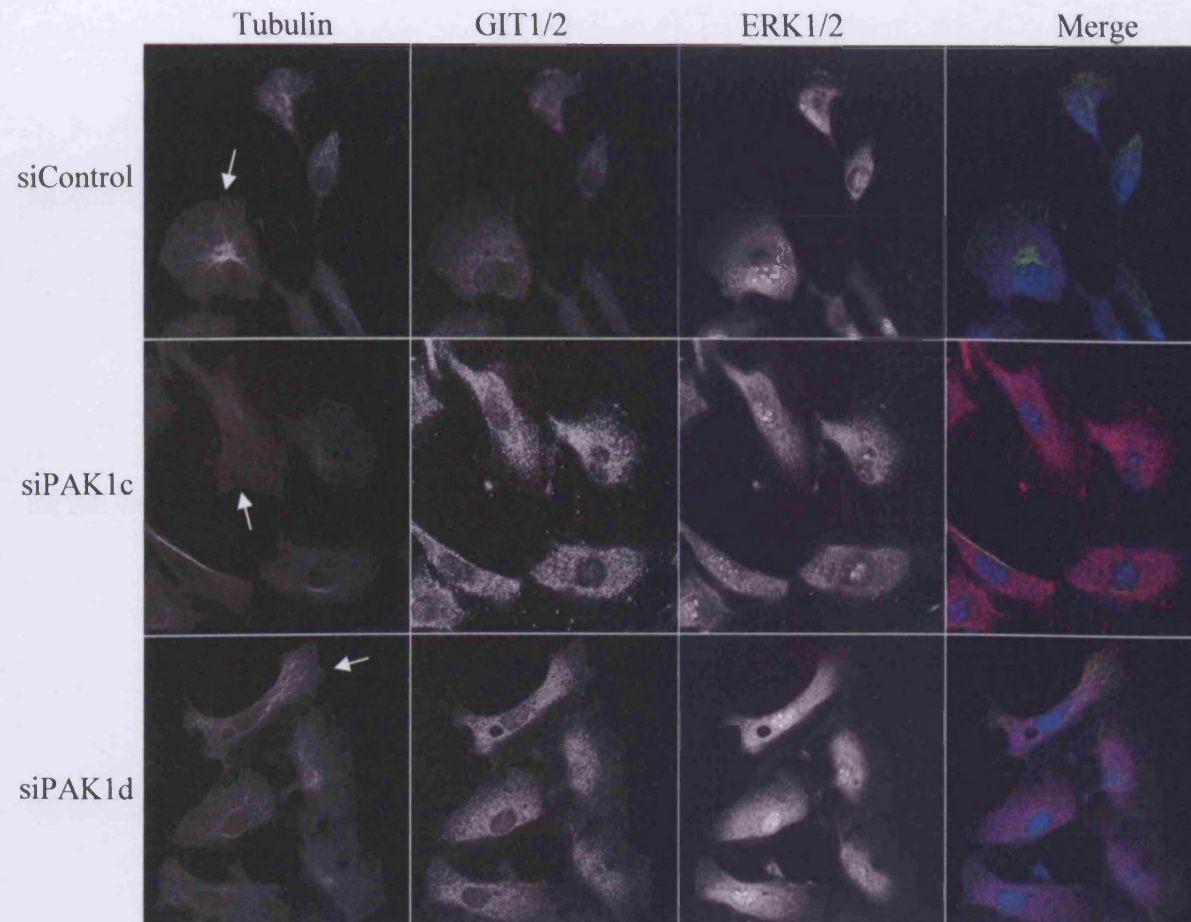


Figure 5.5: Effect of PAK1 knockdown on tubulin, GIT1/2 and ERK1/2 localisation after eighteen hours of HGF stimulation. DU145 cells were transfected with control siRNA or one of two siRNA oligomers against PAK1. 48 hours after transfection, cells were stimulated with 10ng/ml HGF for 18 hours, cells were fixed in methanol and stained with antibodies against GIT1/2 and ERK1/2 followed by fluorescent secondary antibodies and a FITC-conjugated tubulin antibody. Arrows indicate microtubules oriented towards the edges of lamellipodia.

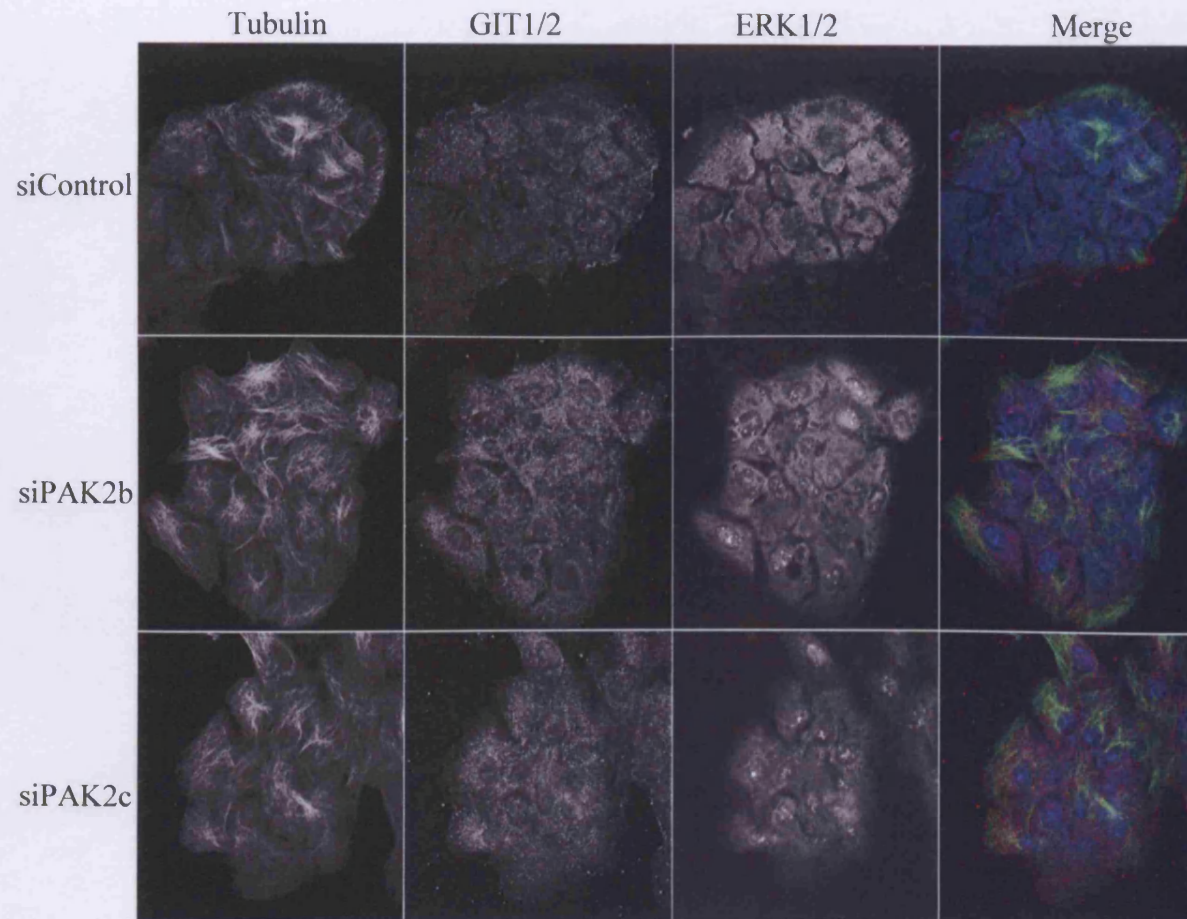


Figure 5.6: Effect of PAK2 knockdown on tubulin, GIT1/2 and ERK1/2 localisation in growing cells. DU145 cells were transfected with control siRNA or one of two siRNA oligomers against PAK2. 66 hours after transfection, cells were fixed in methanol and stained with antibodies against GIT1/2 and ERK1/2 followed by fluorescent secondary antibodies and a FITC-conjugated tubulin antibody. Note that control images are the same as in Figure 5.4 as they are from the same experiment.

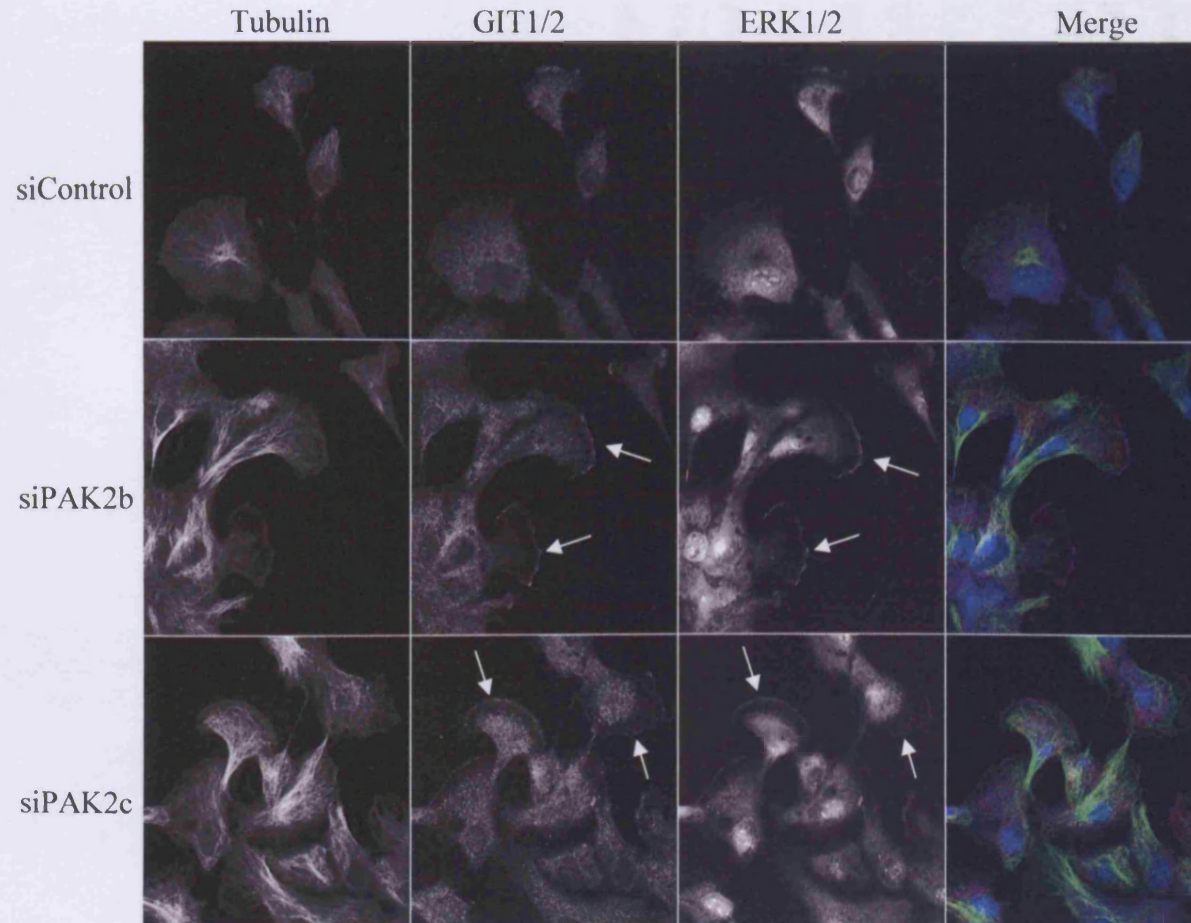


Figure 5.7: Effect of PAK2 knockdown on tubulin, GIT1/2 and ERK1/2 localisation eighteen hours of HGF stimulation. DU145 cells were transfected with control siRNA or one of two siRNA oligomers against PAK2. 48 hours after transfection, cells were stimulated with 10ng/ml HGF for 18 hours, fixed in methanol and stained with antibodies against GIT1/2 and ERK1/2 followed by fluorescent secondary antibodies and a FITC-conjugated tubulin antibody. Control images are the same as in Figure 5.5 as they are from the same experiment. Arrows indicate enhanced localisation of ERK1/2 and GIT1/2 to the edges of lamellipodia.

5.5: Knockdown of PAK2 impairs phosphorylation of Op18 at Serine 16 in PC3 cells but not in DU145 cells

Knockdown of PAK1 or PAK2 impaired the migration of PC3 cells causing them to move slower on average than control cells (Chapter 4). This could be caused by a defect in the establishment or persistence of cell polarity. Microtubule organisation also appeared to be affected by PAK2 knockdown in DU145 cells (Figure 5.6). Microtubules contribute to cell polarity and PAK activation in the leading edge of cells is thought to affect this by phosphorylation and inhibition of Op18/stathmin, a microtubule-destabilising protein (Howell et al., 1999; Marklund et al., 1996). To investigate whether the migration defect in PAK1 or PAK2 deficient PC3 cells could be due to impaired cell polarity, the effect of PAK1 or PAK2 knockdown on phosphorylation of Op18 was observed by western blotting (Figures 5.8 and 5.9). Op18 was phosphorylated in response to HGF in both DU145 and PC3 cells and knockdown of PAK1 did not affect this in either cell line. PAK2 knockdown did not affect Op18 phosphorylation in DU145 cells but in PAK2-depleted PC3 cells the phosphorylation of Op18 was decreased (Figure 5.9 and 5.10). It would be interesting to test later time points to investigate whether there is an effect in DU145 cells later after HGF stimulation.

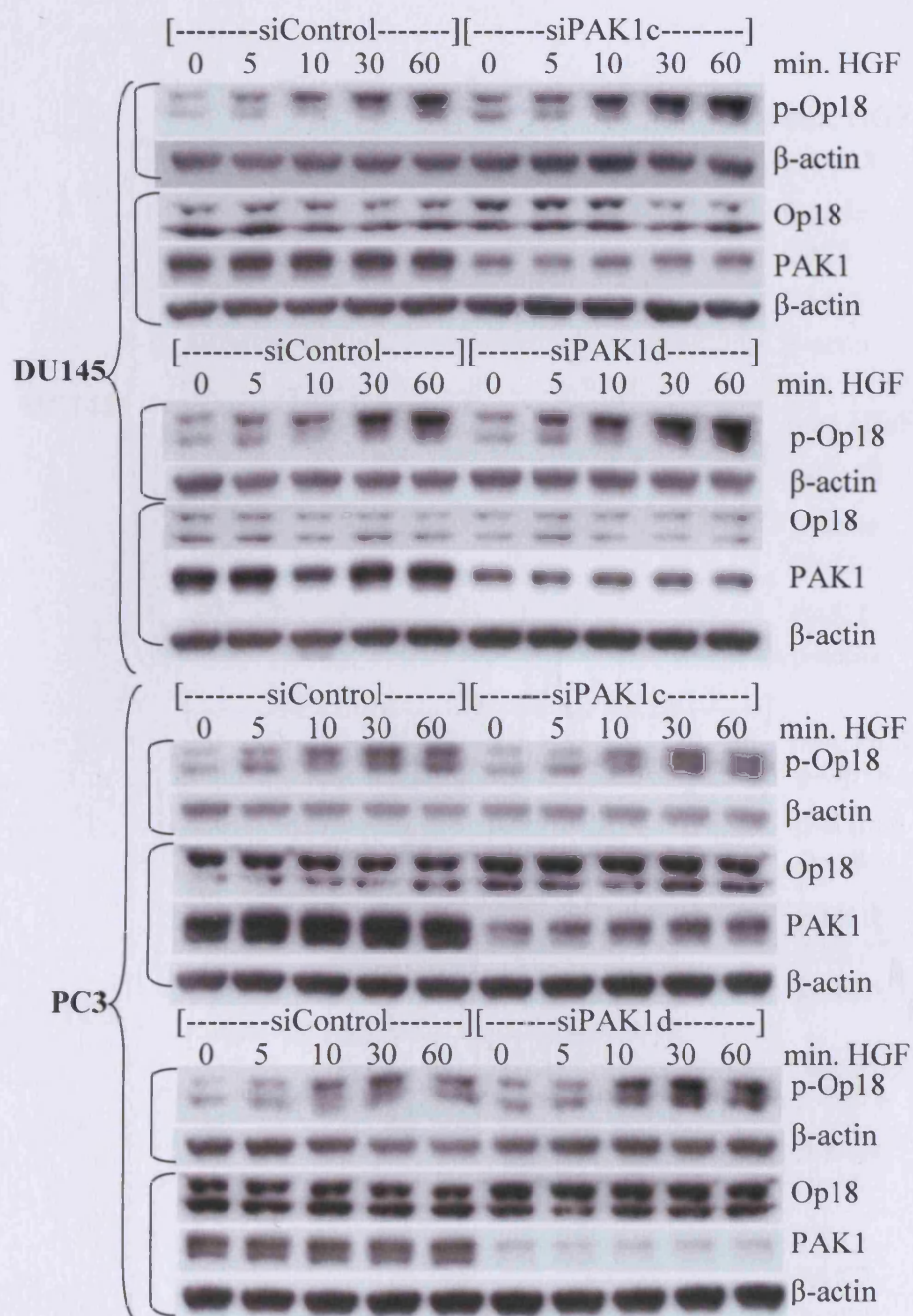


Figure 5.8: PAK1 knockdown does not affect the phosphorylation of Op18 after HGF stimulation. DU145 or PC3 cells were transfected with control siRNA or one of two siRNA oligomers against PAK1. Forty-eight hours after transfection, cells were stimulated with 10 ng/ml HGF for 0 – 60 minutes before lysates were prepared. Western blots were performed and probed with antibodies against phospho-Op18 (pS16) and β-actin, or Op18, PAK1 and β-actin. Control blots are the same as in Figure 5.25. Straight left brackets indicate images from the same membrane. Blots are representative of two independent experiments.

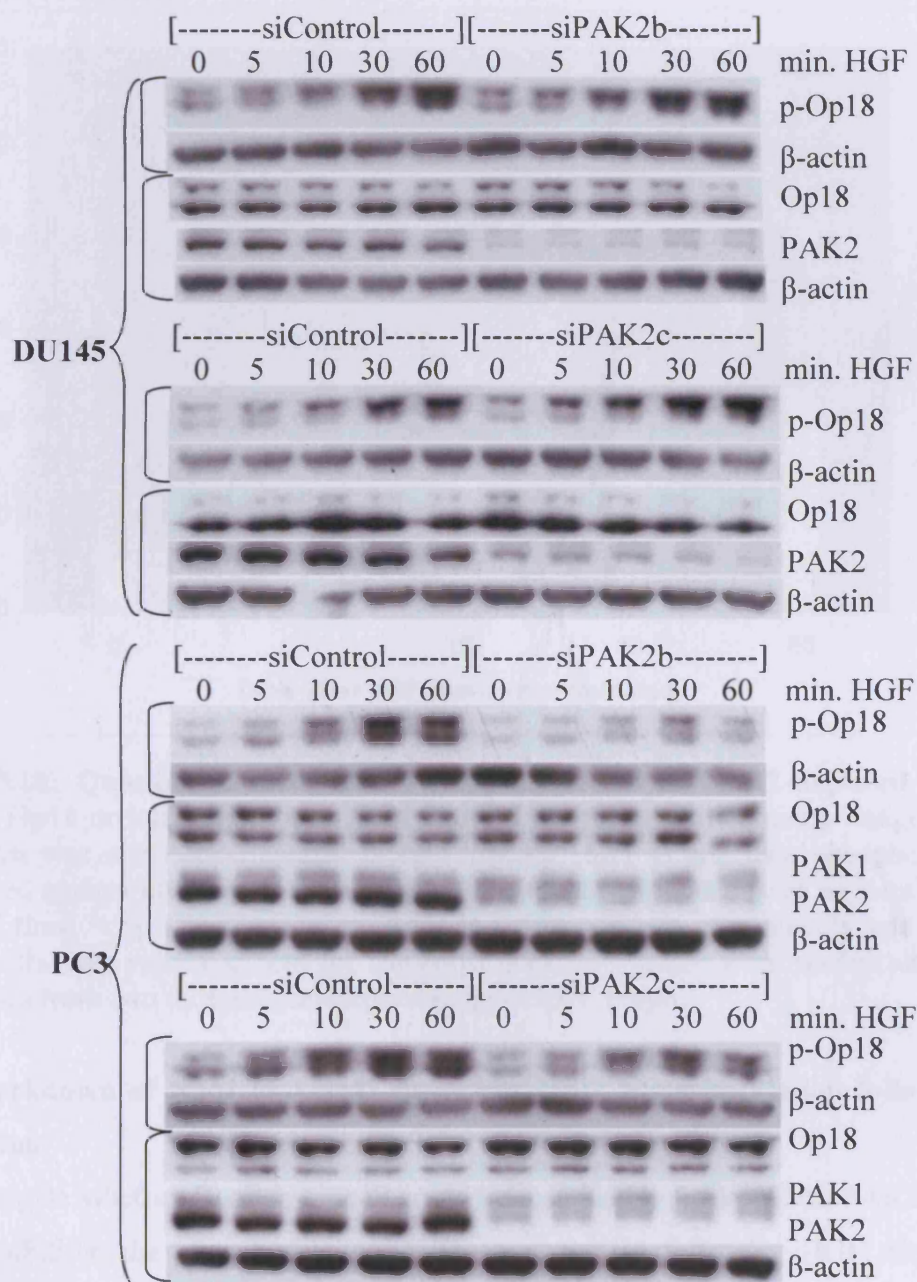


Figure 5.9: PAK2 knockdown impairs the phosphorylation of Op18 after HGF stimulation in PC3 cells but not DU145 cells. DU145 or PC3 cells were transfected with control siRNA or one of two siRNA oligomers against PAK2. Forty-eight hours after transfection, cells were stimulated with 10 ng/ml HGF for 0 – 60 minutes before lysates were prepared. Western blots were performed and probed with antibodies against phospho-Op18 (S16) and β-actin or Op18, PAK2 and actin (DU145) or Op18, PAK1, PAK2 and β-actin (PC3). Straight left brackets indicate images from the same membrane. Some control bands are the same between Figures 3.11 3.13, 5.3, 5.9, 5.18, 5.22, 5.24 and 5.26. Blots are representative of two independent experiments.

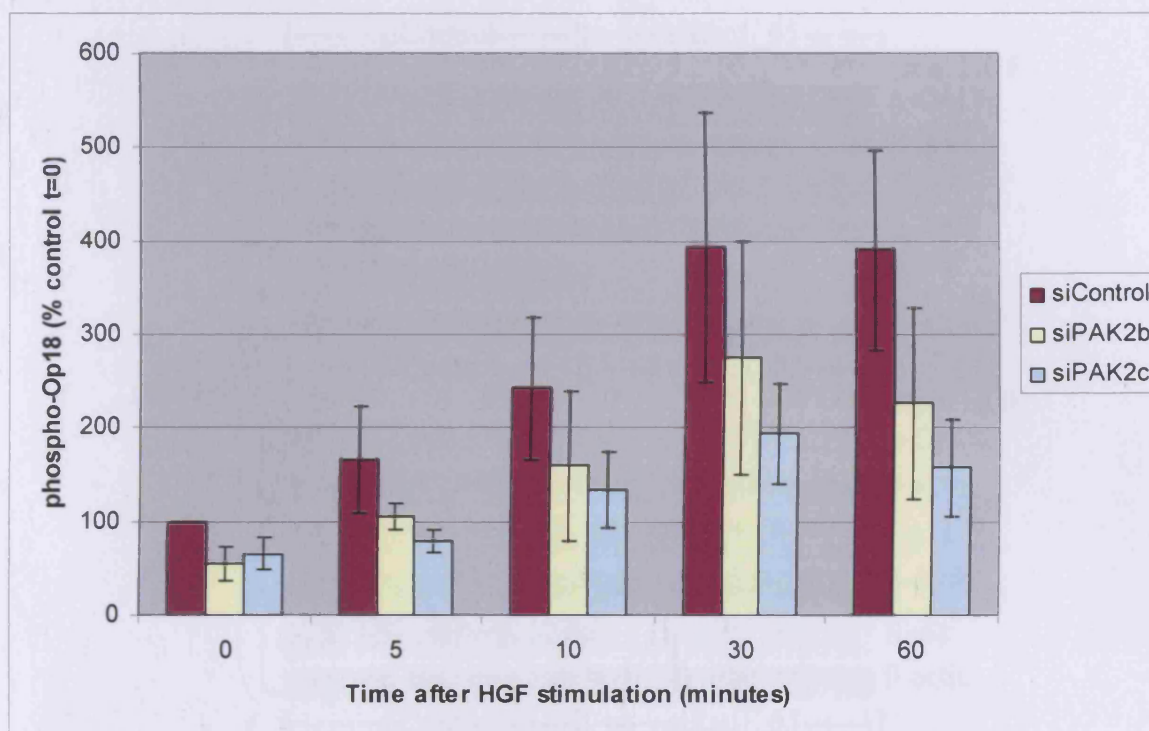


Figure 5.10: Quantification of Op18 phosphorylation in PAK2-depleted PC3 cells. phospho-Op18 or total Op18 blots from figure 5.9 were quantified using ImageJ software. First, each was normalised against β -actin loading controls and then phospho-Op18 was normalised against total Op18. The level of phospho-Op18 is expressed as % of the control value at time = 0. Data are taken from two independent experiments and error bars represent the two values, except for control experiments which were pooled and represent four values from two independent experiments for each graph.

5.6: Knockdown of Rac1 or Cdc42 decreases Op18 phosphorylation following HGF stimulation

To investigate whether Rac1 or Cdc42 could be responsible for PAK1 or PAK2-dependent Op18 inhibition, the phosphorylation of Op18 at Ser 16 following HGF stimulation of Rac1-depleted (Figure 5.11) or Cdc42-depleted (5.13) DU145 or PC3 cells was observed by western blotting. Knockdown of either Rac1 or Cdc42 decreased Op18 phosphorylation following HGF stimulation although the effect was weak for oligomer Rac1_05. More experimental repeats are required to determine the significance of this (Figure 5.12). The effect was strongest in Cdc42-depleted PC3 cells where both oligomers gave a significant decrease in Op18 phosphorylation in two separate experiments (Figure 5.14).

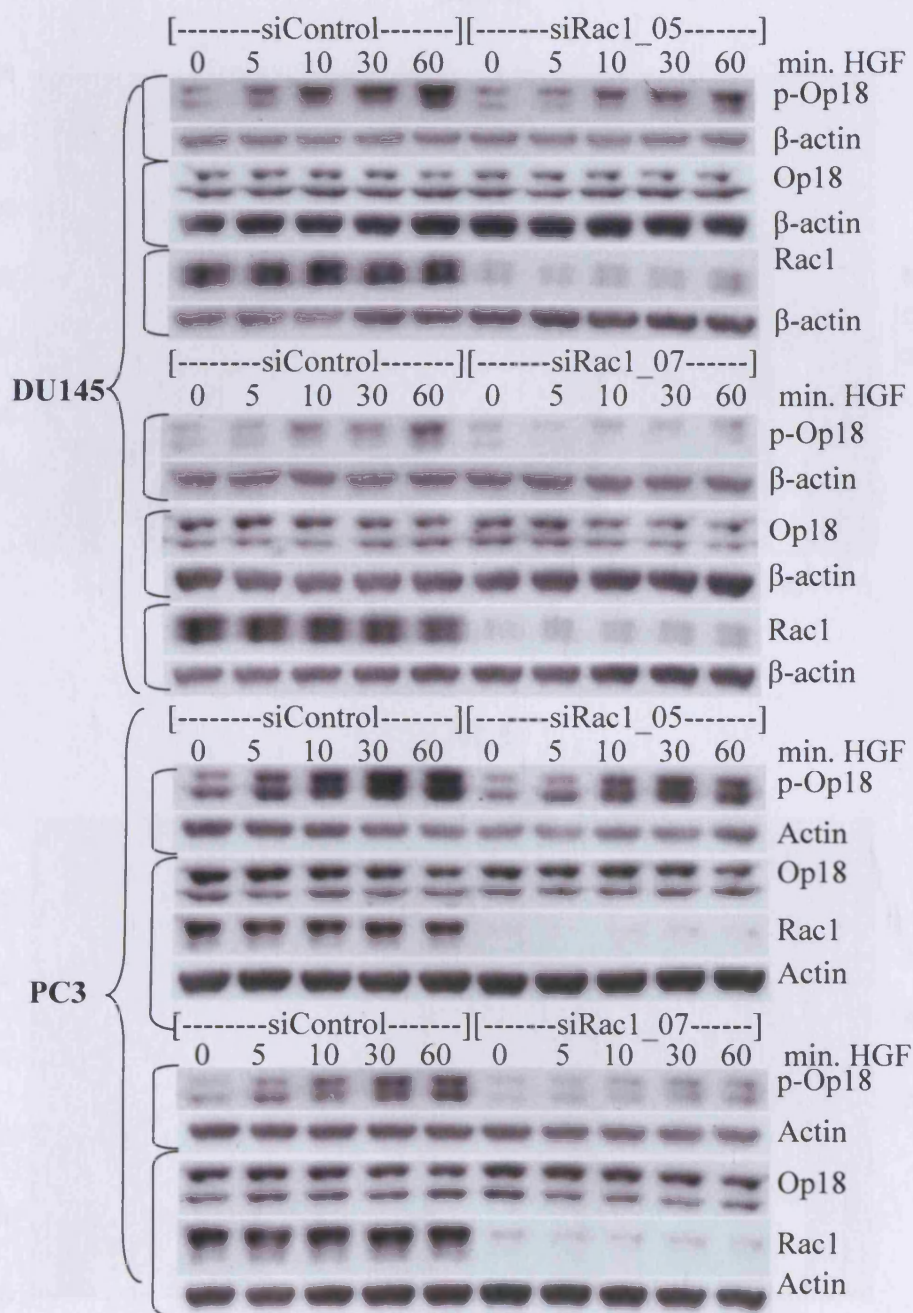


Figure 5.11: Rac1 knockdown impairs the phosphorylation of Op18 after HGF stimulation in PC3 and DU145 cells. DU145 or PC3 cells were transfected with control siRNA or one of two siRNA oligomers against Rac1. Forty-eight hours after transfection, cells were stimulated with 10 ng/ml HGF for 0 – 60 minutes before lysates were prepared. Western blots were performed and probed with antibodies against phospho-Op18 (S16) and β-actin, or Op18 and β-actin, or Rac1 and β-actin (DU145), or Op18, Rac1 and β-actin (PC3). Blots are representative of three independent experiments. Straight left brackets indicate images from the same membrane.

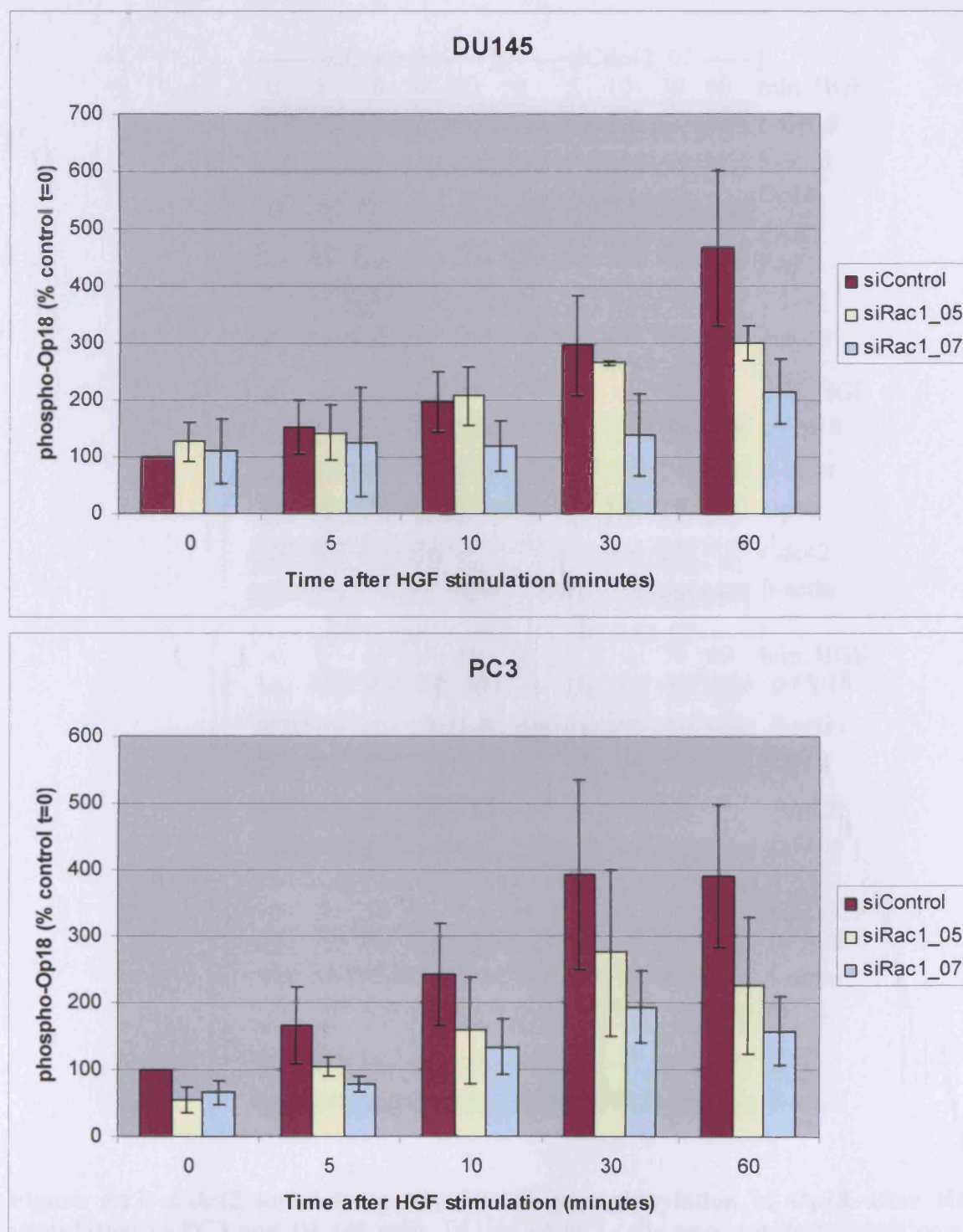


Figure 5.12: Op18 phosphorylation is impaired in Rac1-depletion in DU145 and PC3 cells. phospho-Op18 or total Op18 blots from figure 5.11 were quantified using ImageJ software. First, each was normalised against β -actin loading control and then phospho-Op18 was normalised against total Op18. The level of phospho-Op18 is expressed as % of the control value at time = 0. Data are taken from two independent experiments and error bars represent the two means, except for control experiments which were pooled and represent four means from two independent experiments for each graph.

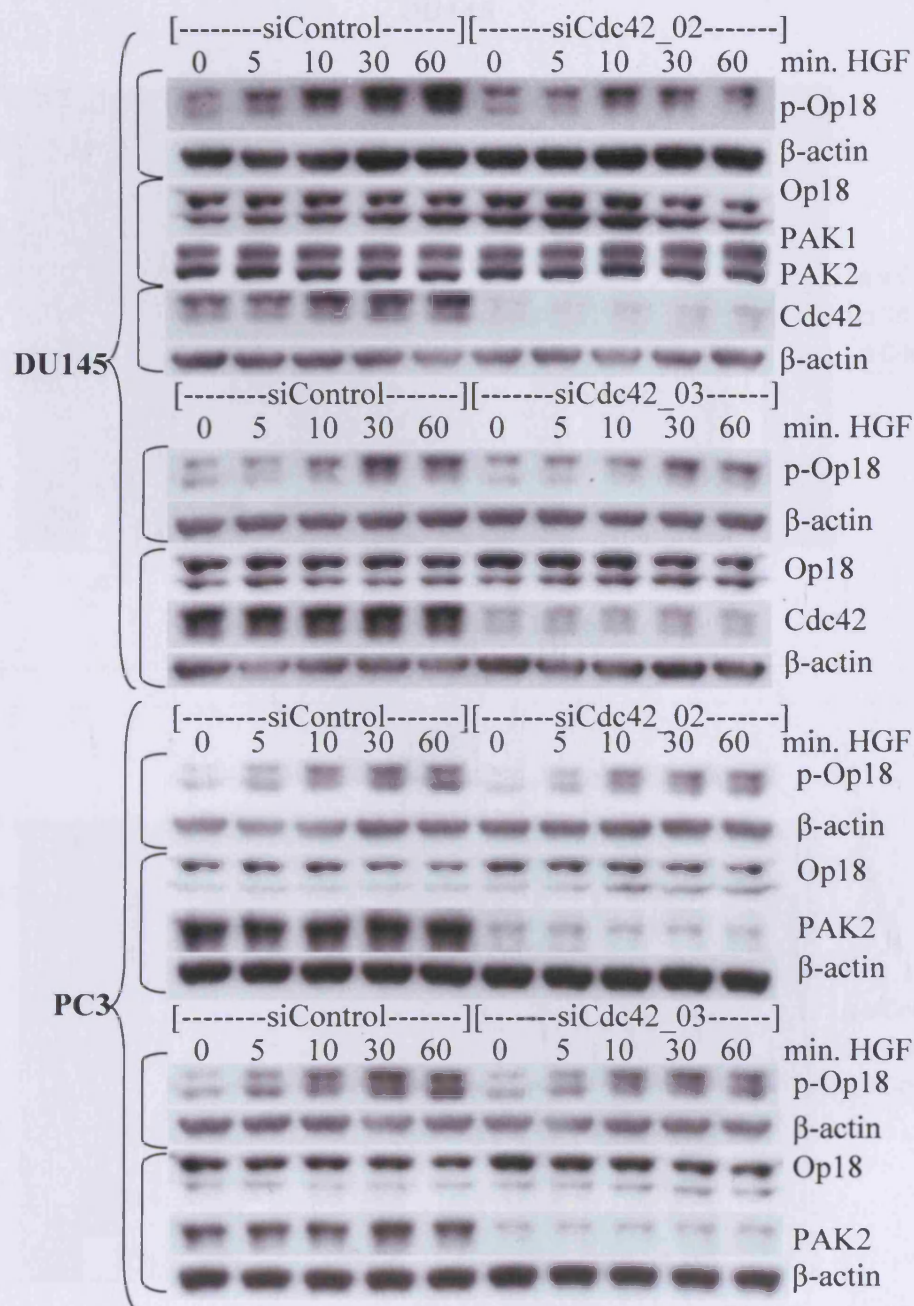


Figure 5.13: Cdc42 knockdown impairs the phosphorylation of Op18 after HGF stimulation in PC3 and DU145 cells. DU145 or PC3 cells were transfected with control siRNA or one of two siRNA oligomers against Cdc42. Forty-eight hours after transfection, cells were stimulated with 10 ng/ml HGF for 0 – 60 minutes before lysates were prepared. Western blots were performed and probed with antibodies against phospho-Op18 (S16) and β -actin, or PAK1, PAK2 and Op18, or Cdc42 and β -actin (top panel), or Op18, Cdc42 and β -actin. Blots are representative of three independent experiments. Straight left brackets indicate images from the same membrane.

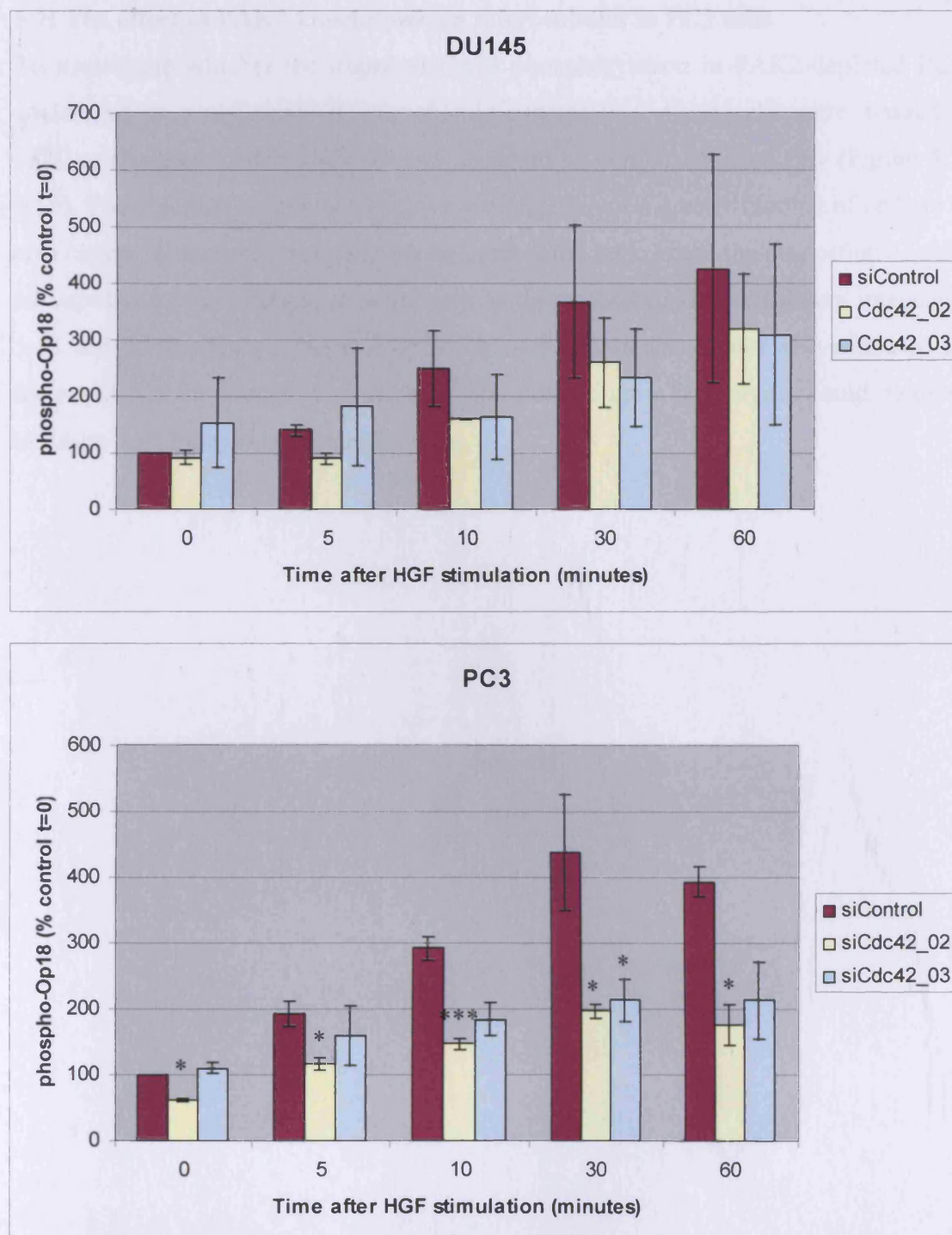


Figure 5.14: Quantification of Op18 phosphorylation Cdc42-depleted DU145 and PC3 cells. phospho-Op18 or total Op18 blots from figure 5.13 were quantified using ImageJ software. First, each was normalised against β -actin loading controls and then phospho-Op18 was normalised against total Op18. The level of phospho-Op18 is expressed as % of the control value at time = 0. Data are taken from two independent experiments and error bars represent the two means except for control experiments which were pooled and represent four means from two independent experiments for each graph.. * = $p < 0.05$, *** = $p < 0.001$, two tailed student's t-test.

5.7: The effect of PAK2 knockdown on microtubules in PC3 cells

To investigate whether the impaired Op18 phosphorylation in PAK2-depleted PC3 cells could lead to a difference in microtubule organisation, fixed cells were stained with a FITC-conjugated tubulin antibody and observed by confocal microscopy (Figure 5.15 and 5.16). These results require quantitative investigation of a greater number of cells to draw a conclusion. A method for carrying out analysis could be to count the proportion of cells with an unpolarised microtubule network such as those observed in preliminary images (Figure 5.15 and 5.16, arrows). The cell periphery of PAK2-depleted cells also appeared to have increased F-actin compared to control cells. Both of these phenotypes could be quantified in future with more experimental repeats.

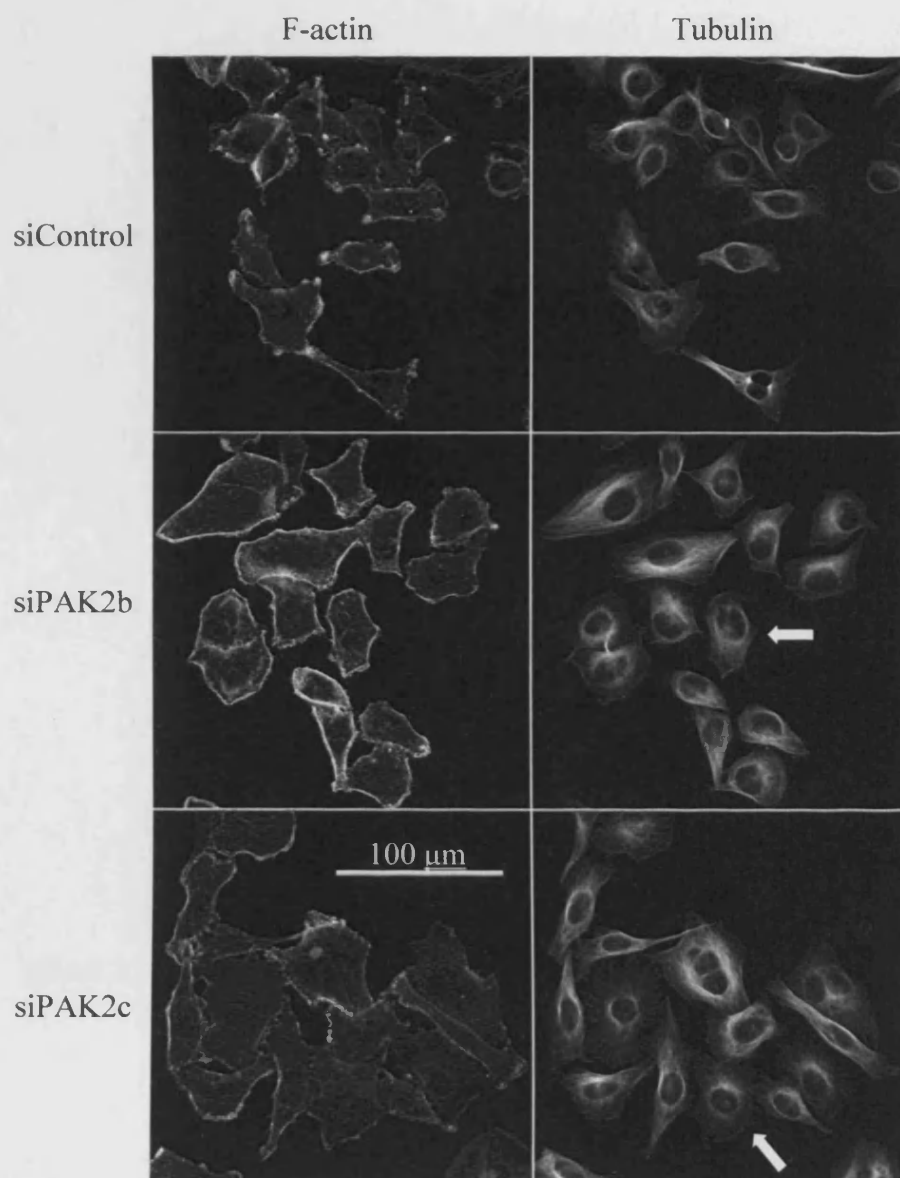


Figure 5.15: Effect of PAK2 knockdown on PC3 microtubule organisation in unstimulated cells. PC3 cells were transfected with control siRNA or siRNA against one of two PAK2 oligomers using Lipofectamine 2000. 66 hours after transfection cells were fixed in 4% paraformaldehyde and stained with Alexa 546-linked phalloidin and a FITC-conjugated anti-tubulin antibody. Confocal images were captured using a 40X oil-immersion objective. Arrows indicate examples of cells without apparent microtubule network polarisation. Data are from one experiment.

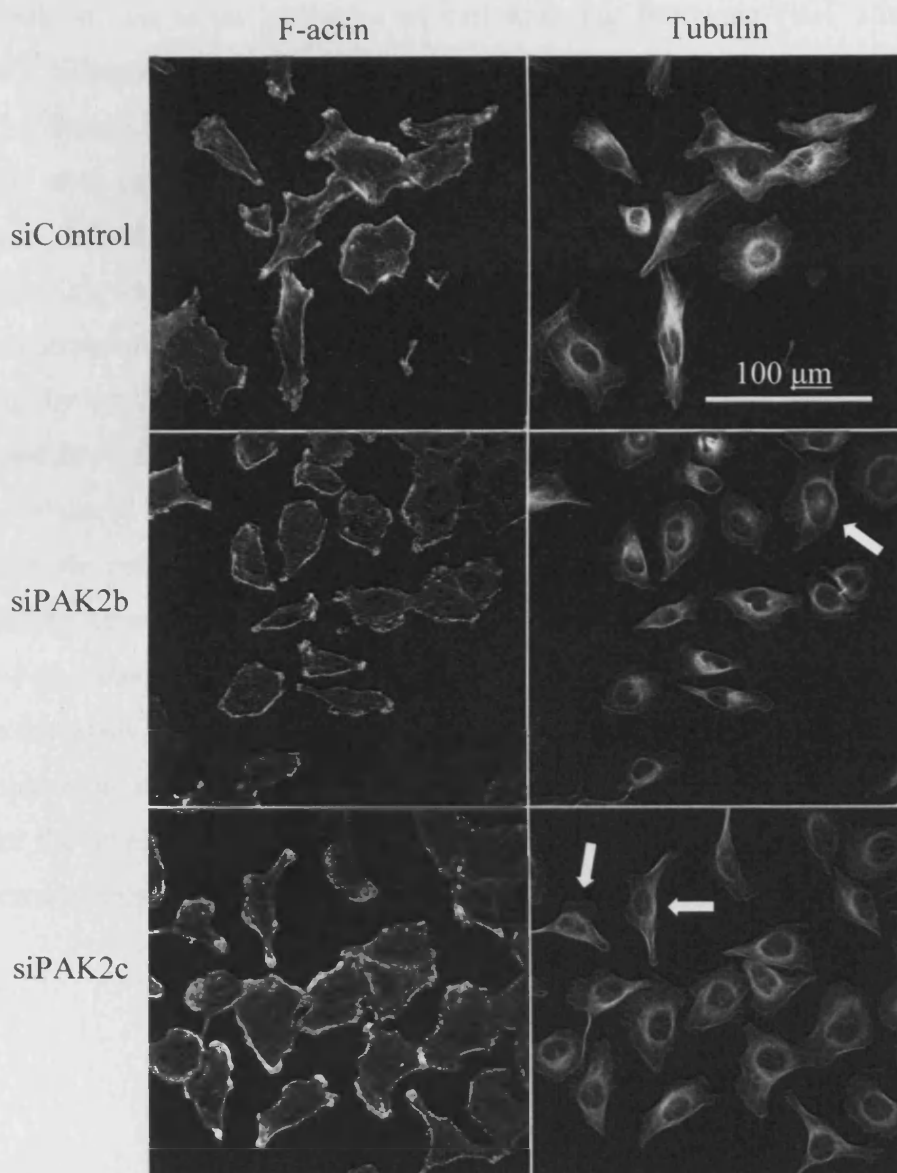


Figure 5.16: Effect of PAK2 knockdown on PC3 microtubule organisation in unstimulated cells: PC3 cells were transfected with control siRNA or siRNA against one of two PAK2 oligomers using Lipofectamine 2000. 48 hours after transfection cells were stimulated with 10 ng/ml HGF for 18 hours, then fixed in 4% paraformaldehyde and stained with Alexa 546-linked phalloidin and a FITC-conjugated anti-tubulin antibody. Confocal images were captured using a 40X oil-immersion objective. Arrows indicate examples of cells without apparent microtubule network polarisation. Data are from one experiment.

5.8: The effect of knockdown of PAK1 or PAK2 on MLC phosphorylation

PAK1 knockdown led to an inhibition of cell rounding following HGF stimulation in DU145 cells (Chapter 4, Section 4.3). This could be due to a decrease in myosin contractility. Myosin contraction is controlled by phosphorylation of R-MLC at Thr18 and Ser19. These sites can be phosphorylated by MLCK (both T18 and S19), ROCK (S19) (Amano et al., 1996) and PAK (S19) (Chew et al., 1998; Ramos et al., 1997). PAK can also phosphorylate and inactivate MLCK (Goeckeler et al., 2000). The effect of PAK1 or PAK2 knockdown on the level of diphosphorylated R-MLC following HGF stimulation was observed by western blotting (Figures 5.17 and 5.18). Preliminary results using lysates from PC3 and DU145 cells suggested that HGF stimulation did not affect the level of R-MLC phosphorylated at both T18 and S19 up to 1 hour after HGF addition, and the level also remained the same following knockdown of PAK1 or PAK2. However, there was some variability between western blots so the results are not conclusive and should be repeated. No consistent change in R-MLC phosphorylation following HGF stimulation was observed in this assay, it is possible that there could be a difference at later time points after HGF stimulation as cell rounding was most apparent after 16 hours or more. It is also possible that the level of monophosphorylated R-MLC could change but would probably not be detected by this antibody.

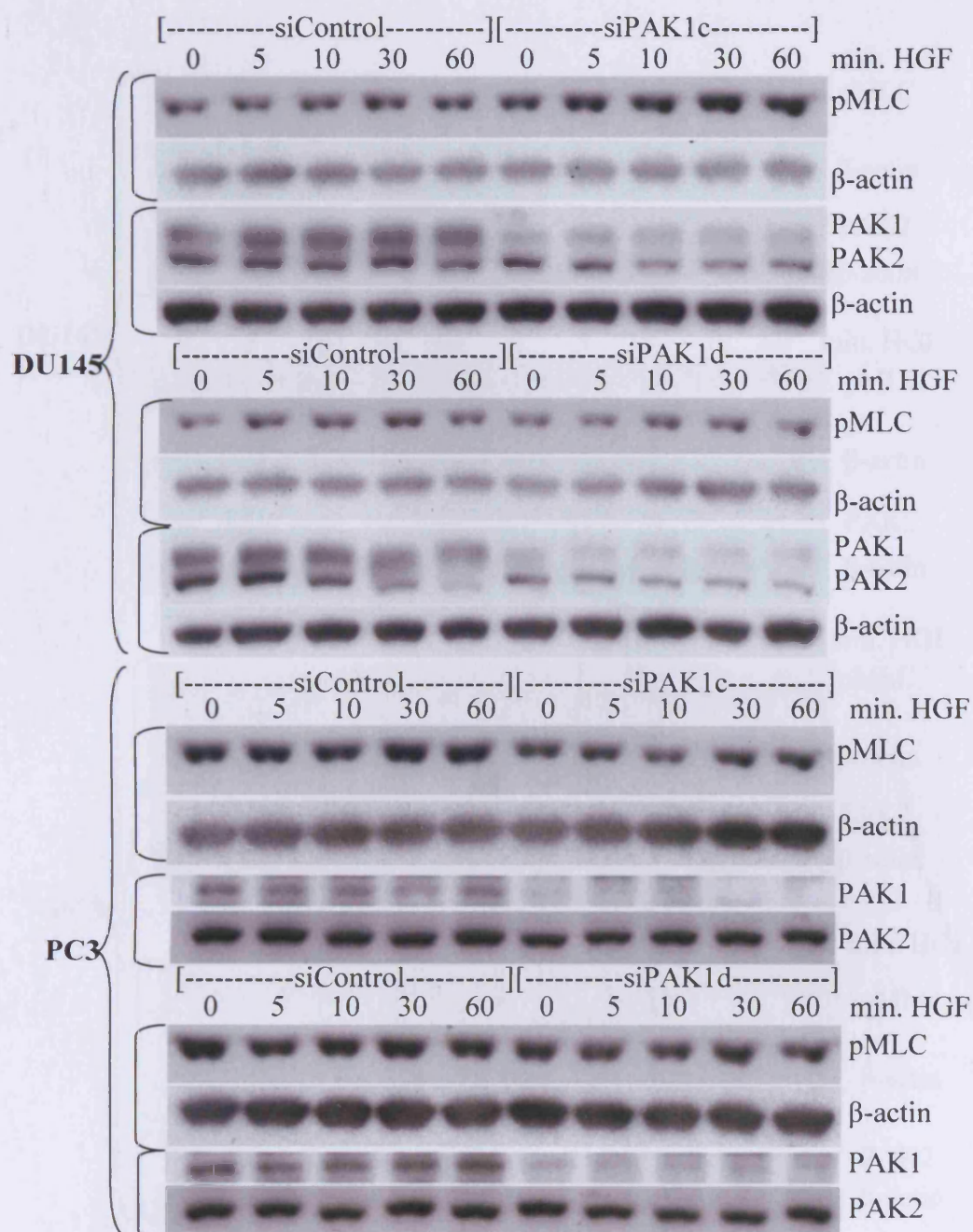


Figure 5.17: PAK1 knockdown does not affect the phosphorylation of MLC downstream of HGF. DU145 or PC3 cells were transfected with control siRNA or one of two siRNA oligomers against PAK1. Forty-eight hours after transfection, cells were stimulated with 10 ng/ml HGF for 0 – 60 minutes before lysates were prepared. Western blots were performed and probed with antibodies against phospho-MLC (T18/S19) and β -actin or PAK1, PAK2 and β -actin (DU145) or PAK1 and PAK2 (PC3). Straight left brackets indicate images from the same membrane. Some control blots are the same between Figures 3.10, 3.12, 5.2, 5.17, 5.23 and 5.21. Blots are from one experiment for pMLC.

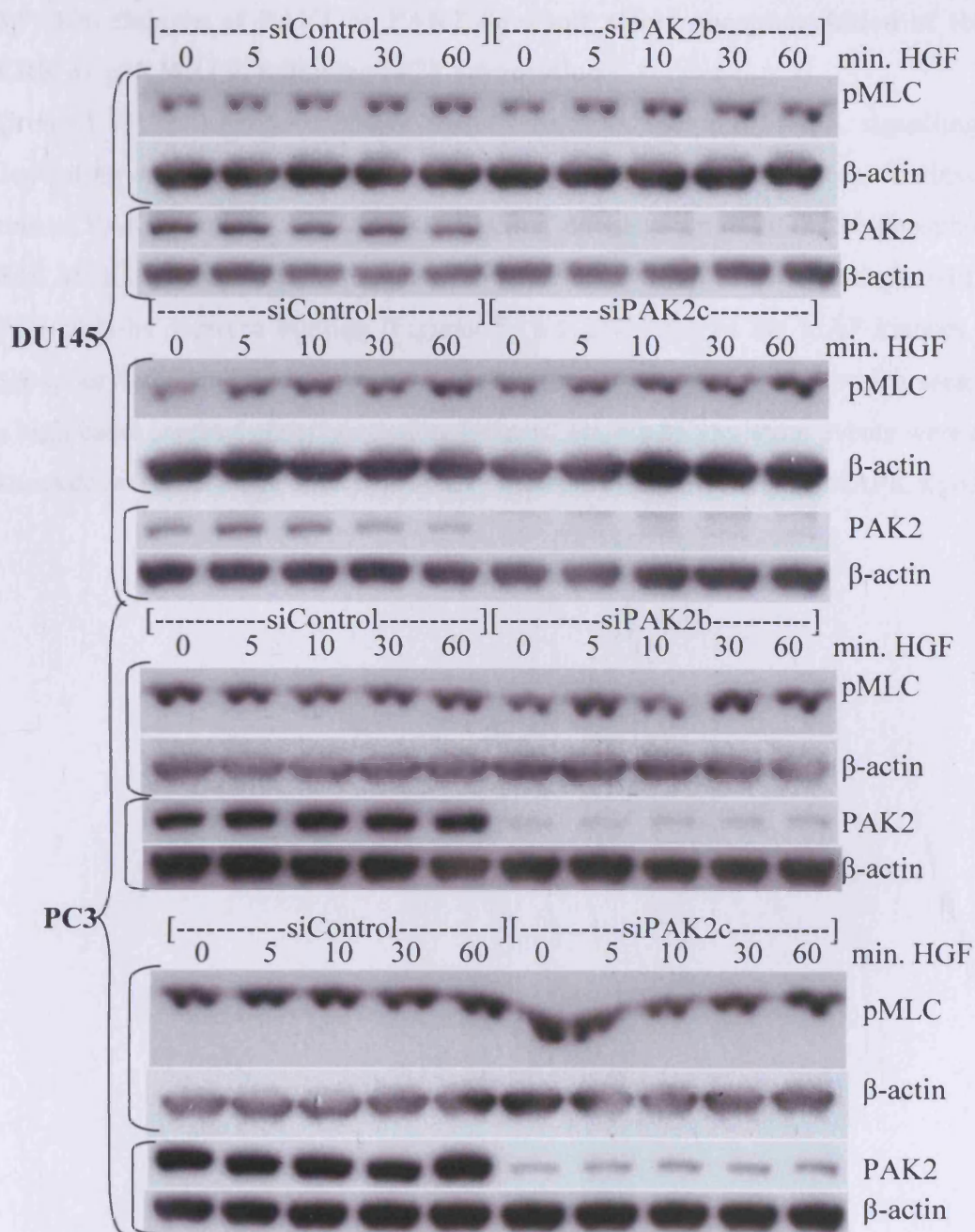


Figure 5.18: PAK2 knockdown does not affect the phosphorylation of MLC downstream of HGF. DU145 or PC3 cells were transfected with control siRNA or one of two siRNA oligomers against PAK2. Forty-eight hours after transfection, cells were stimulated with 10 ng/ml HGF for 0 – 60 minutes before lysates were prepared. Western blots were performed and probed with antibodies against phospho-MLC (T18/S19) and β -actin or PAK2 and β -actin. Straight left brackets indicate images from the same membrane. Some control bands are the same between Figures 3.11 3.13, 5.9, 5.18, 5.22 and 5.24. Blots represent one experiment for pMLC.

5.9: Knockdown of PAK1 or PAK2 does not affect phosphorylation of Raf, MEK1, ERK or p38 MAPK following HGF stimulation

Group I PAKs have been shown to play roles in multiple MAPK signalling pathways depending on the cell type and stimulus (See Chapter 1, Section 1.7.4). To investigate the role of PAK1 and PAK2 in MAPK signalling downstream of HGF, the phosphorylation of Raf, MEK1, ERK and p38 MAPK were observed in PAK1 or PAK2-depleted DU145 and PC3 cells by western blotting (Figures 5.19-5.22). Each of the MAP kinases tested was phosphorylated rapidly following HGF stimulation except for MEK which seemed to have a high basal level of phosphorylation. None of the phosphorylation events were affected by knockdown of PAK1 or PAK2, probably because of the diversity of MAPK signalling.

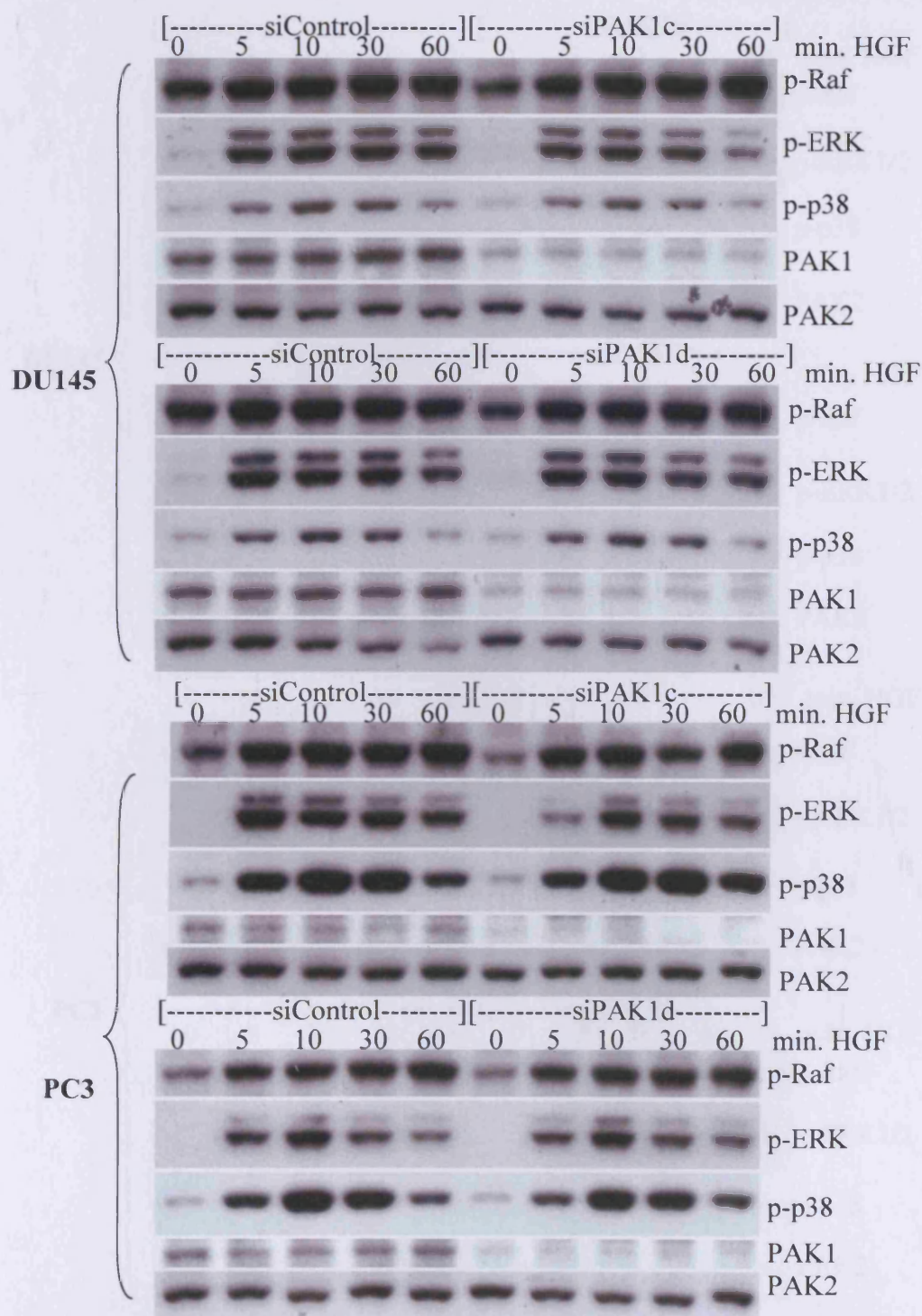


Figure 5.19: The effect of PAK1 knockdown on MAPK signalling. DU145 or PC3 cells were transfected with control siRNA or one of two siRNA oligomers against PAK1. Forty-eight hours after transfection, cells were stimulated with 10 ng/ml HGF for 0 – 60 minutes before lysates were prepared. Western blots were performed and probed with antibodies against phospho-Raf, phospho-ERK (T202/Y204), phospho-p38 MAPK (T180/Y182), PAK1 and PAK2. Some control blots are the same between Figures 3.10, 5.2, 5.17, 5.23 and 5.21. Data are representative of two experiments for pERK1/2 and p-p38, and one experiment for pRaf.

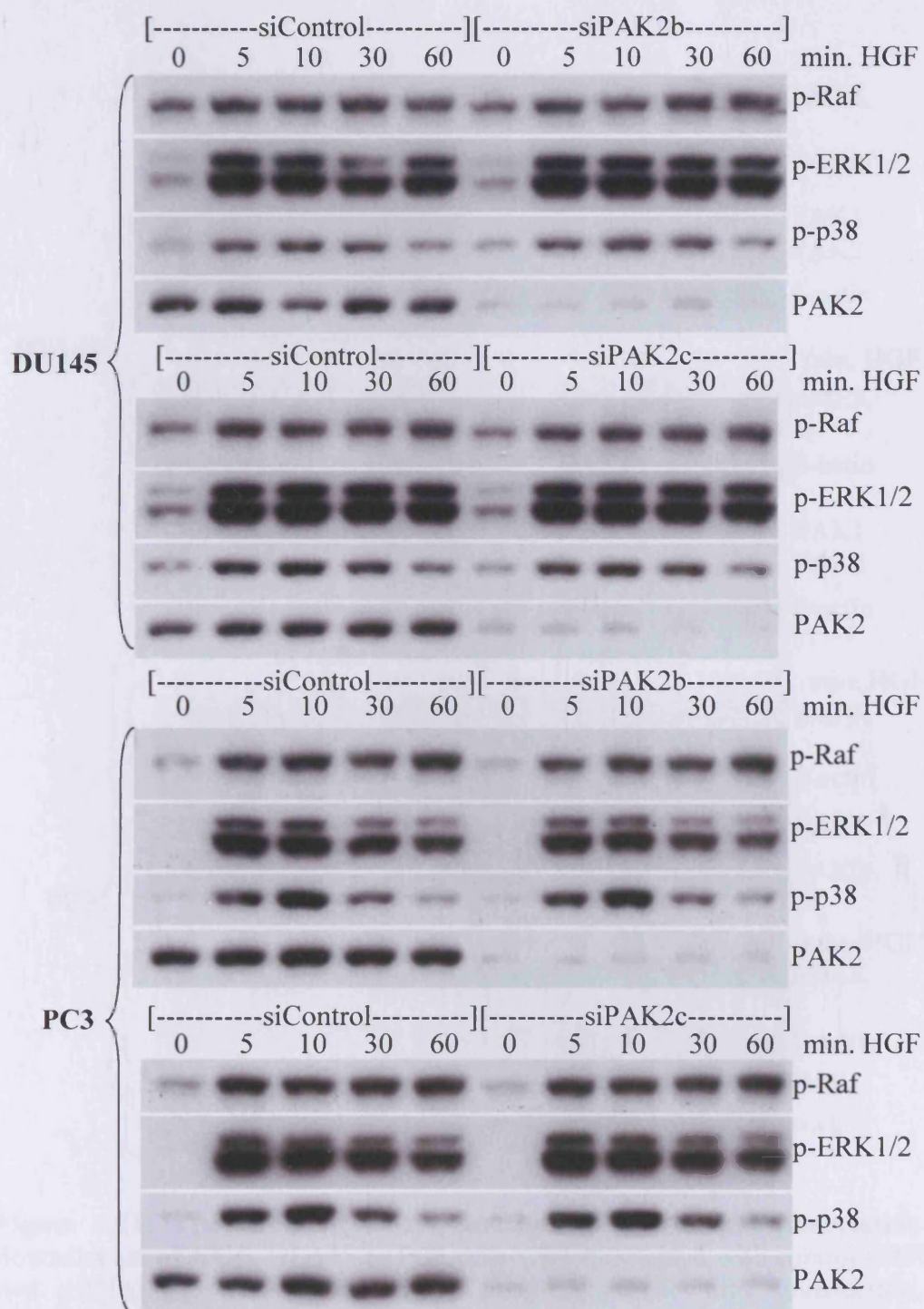


Figure 5.20: The effect of PAK2 knockdown on MAPK signalling. DU145 or PC3 cells were transfected with control siRNA or one of two siRNA oligomers against PAK2. Forty-eight hours after transfection, cells were stimulated with 10 ng/ml HGF for 0 – 60 minutes before lysates were prepared. Western blots were performed and probed with antibodies against phospho-Raf, phospho-ERK1/2 (T202/Y204), phospho-p38 MAPK (T180/Y182) and PAK2. Some control bands are the same between Figures 3.11 3.13, 5.9, 5.18, 5.22 and 5.24. Data are representative of two experiments for pERK1/2 and one experiment for pRaf.

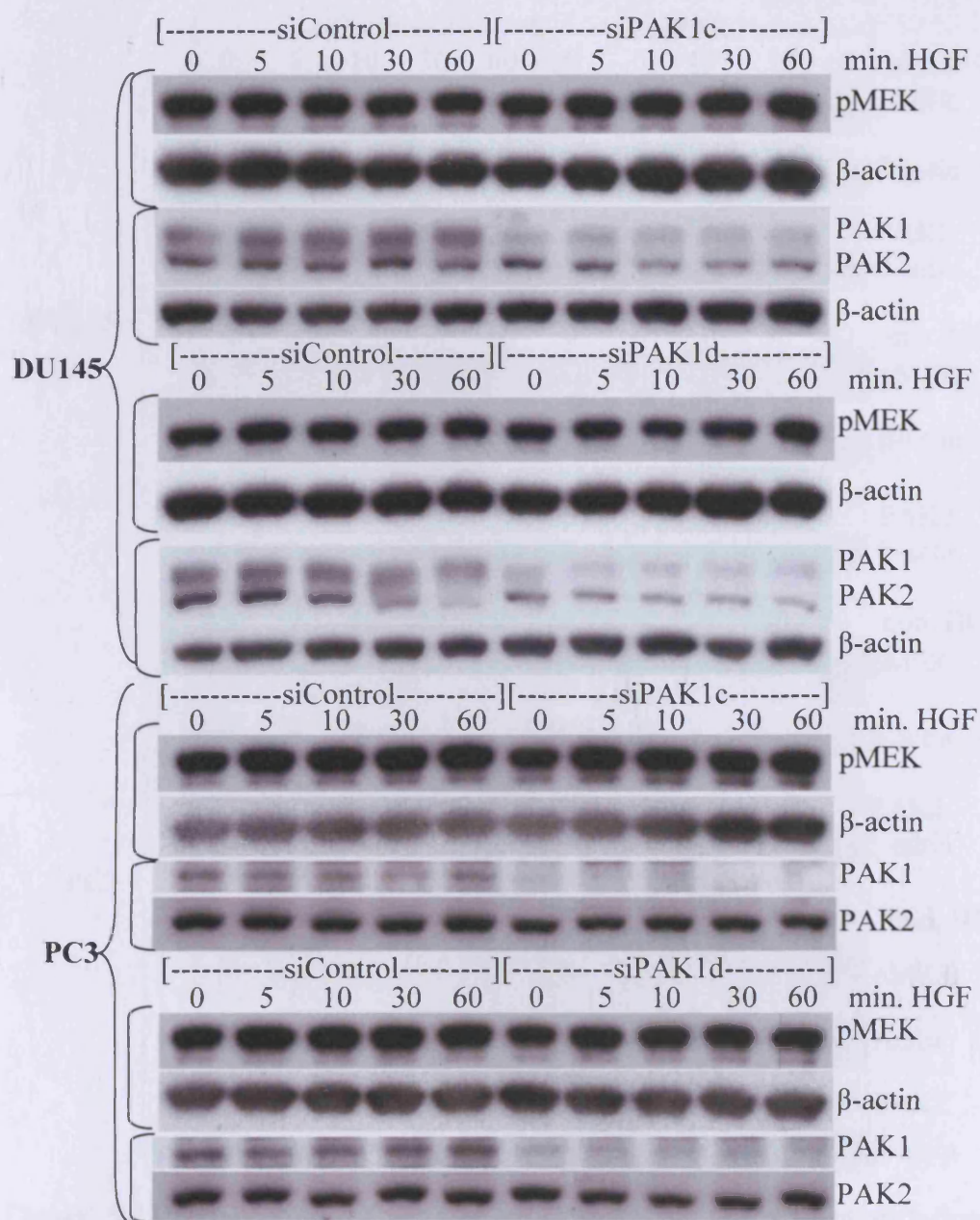


Figure 5.21: The effect of PAK1 knockdown on the phosphorylation of MEK1 downstream of HGF. DU145 or PC3 cells were transfected with control siRNA or one of two siRNA oligomers against PAK1. Forty-eight hours after transfection, cells were stimulated with 10 ng/ml HGF for 0 – 60 minutes before lysates were prepared. Western blots were performed and probed with antibodies against phospho-MEK and β -actin or PAK1, PAK2 and β -actin (DU145), or PAK1 and PAK2 (PC3). Control blots are the same as for Figure 5.2 (PC3) and 5.17 (PC3 and DU145). Straight left brackets indicate images from the same membrane. Some control blots are the same between Figures 3.10, 3.12, 5.2, 5.17, 5.23 and 5.21. Data are from one experiment for pMEK.

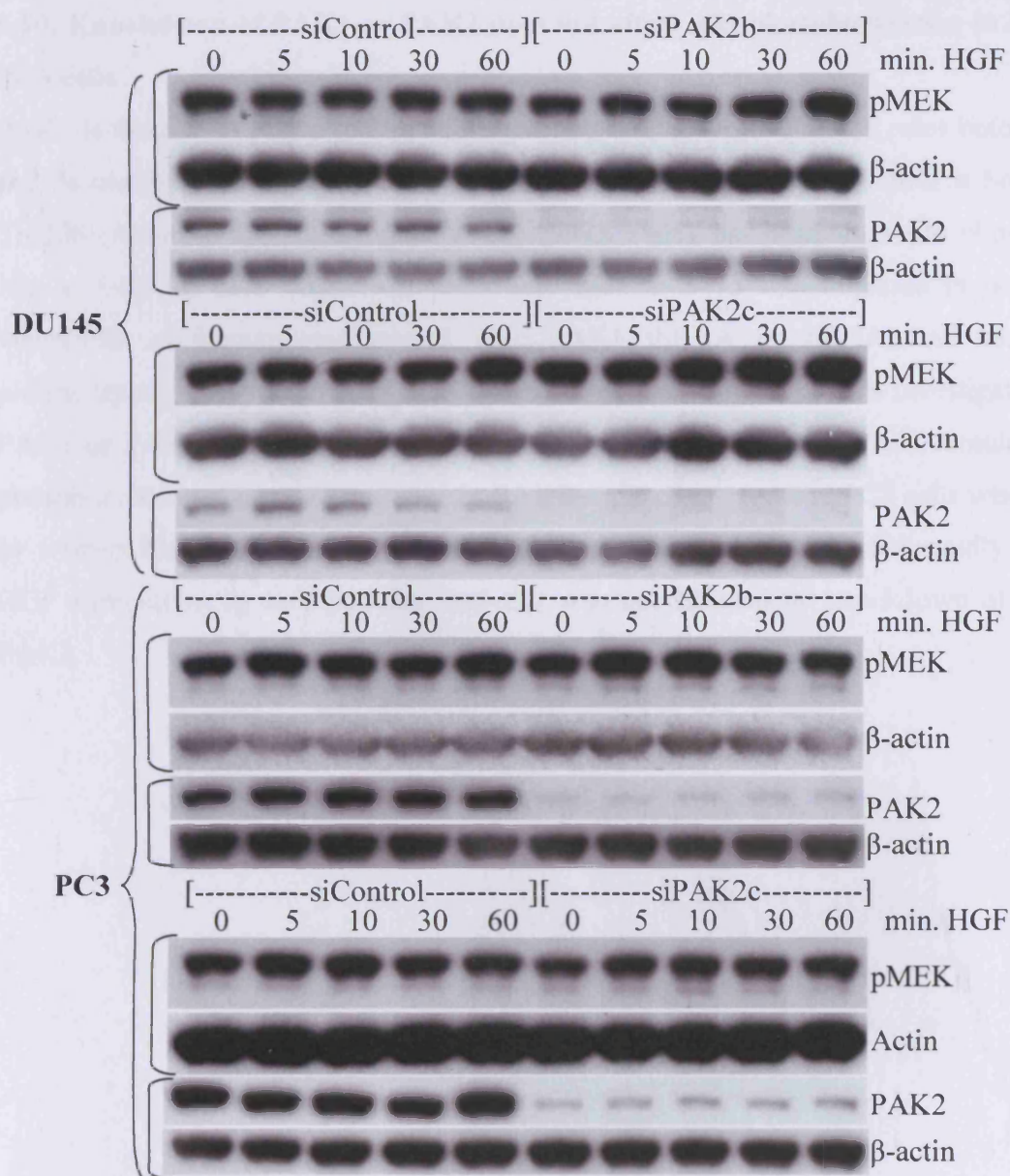


Figure 5.22: The effect of PAK2 knockdown on the phosphorylation of MEK1 downstream of HGF. DU145 or PC3 cells were transfected with control siRNA or one of two siRNA oligomers against PAK2. Forty-eight hours after transfection, cells were stimulated with 10 ng/ml HGF for 0 – 60 minutes before lysates were prepared. Western blots were performed and probed with antibodies against phospho-MEK and β-actin or PAK2 and β-actin. Straight left brackets indicate images from the same membrane. Some control bands are the same between Figures 3.11 3.13, 5.9, 5.18, 5.22 and 5.24. Data are from one experiment for pMEK.

5.10: Knockdown of PAK1 or PAK2 does not affect Akt phosphorylation in DU145 or PC3 cells

PAK1 is thought to play a role in cell signalling through Akt/PKB and roles both upstream and downstream have been described. Akt is activated by phosphorylation at Ser 473 and Thr308 (Alessi et al., 1996; Alessi et al., 1997). PAK1 has been shown to phosphorylate Akt at S473 *in vitro* while Akt phosphorylation at S473 was impaired in neonatal rat ventricular cardiomyocytes treated with PAK1 shRNA or siRNA, and cardiac Akt phosphorylation was reduced in PAK1-null mice (Mao et al., 2008). To investigate whether PAK1 or PAK2 could be responsible for Akt activation following HGF stimulation, Akt phosphorylation at Ser 473 in PAK1 or PAK2-depleted DU145 and PC3 cells was observed by western blotting (Figures 5.23 and 5.24). Akt became phosphorylated rapidly following HGF stimulation in each cell line and this was not affected by knockdown of PAK1 or PAK2.

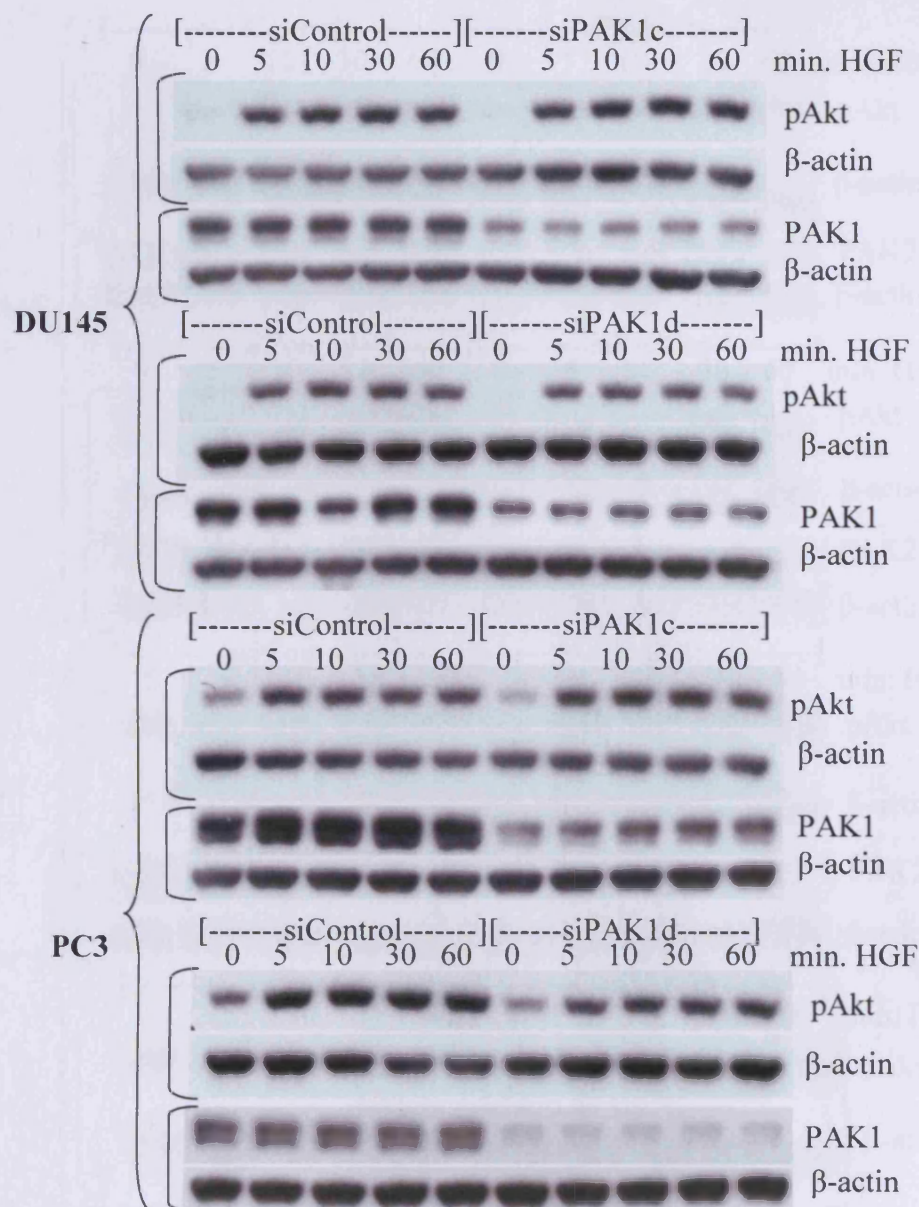


Figure 5.23: PAK1 knockdown does not affect Akt phosphorylation following HGF stimulation. DU145 or PC3 cells were transfected with control siRNA or one of two siRNA oligomers against PAK1. Forty-eight hours after transfection, cells were stimulated with 10 ng/ml HGF for 0 – 60 minutes before lysates were prepared. Western blots were performed and probed with antibodies against phospho-Akt (S473) and β -actin or PAK1 and β -actin. Straight left brackets indicate images from the same membrane. Control blots are the same as in Figure 5.8. Data are representative of two experiments.

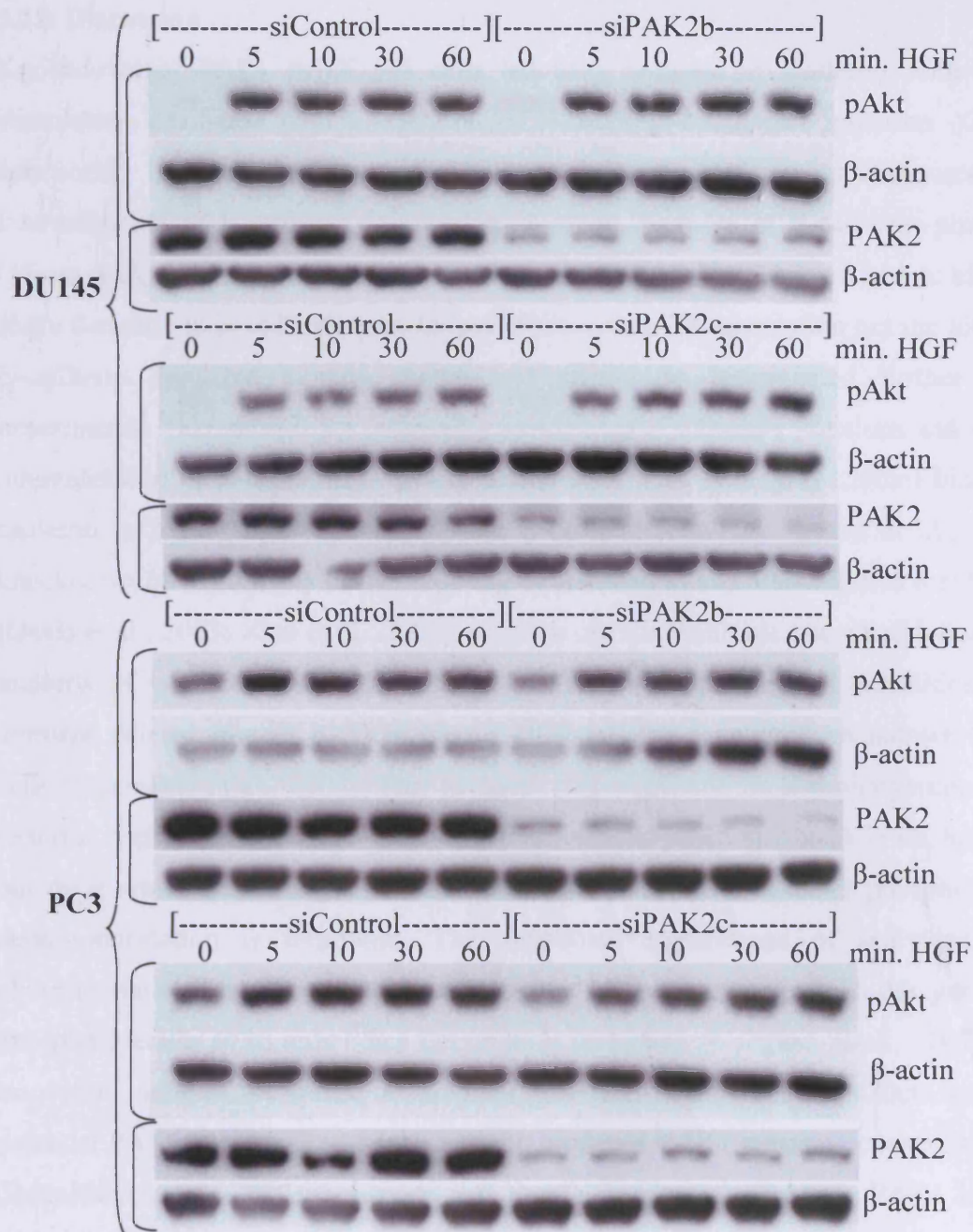


Figure 5.24: PAK2 knockdown does not affect Akt phosphorylation following HGF stimulation. DU145 or PC3 cells were transfected with control siRNA or one of two siRNA oligomers against PAK2. Forty-eight hours after transfection, cells were stimulated with 10 ng/ml HGF for 0 – 60 minutes before lysates were prepared. Western blots were performed and probed with antibodies against phospho-Akt (S473) and β -actin or PAK2 and β -actin. Straight left brackets indicate images from the same membrane. Some control blots are from the same membrane as in Figure 5.3 and 5.9. Data are representative of two experiments.

5.11: Discussion

Knockdown of PAK1 in DU145 cells led to a decrease in scattering following HGF stimulation combined with a retention of E-cadherin-based cell junctions (Chapter 4). Junctional breakdown following HGF stimulation can be characterised by the internalisation of E-cadherin followed by a slight decrease in E-cadherin protein levels (Miura et al., 2001). Preliminary results showed that PAK1 knockdown had no effect on the slight decrease in E-cadherin protein level following HGF stimulation but the total level of E-cadherin appeared slightly higher and should be investigated further in future experiments. The mechanism underlying breakdown of adherens junctions and subsequent internalisation of E-cadherin is poorly understood. p120 catenin (p120ctn) binding to E-cadherin is required for stability of the E-cadherin complex (Ireton et al., 2002) and knockdown of p120 leads to the targeting of E-cadherin and VE-cadherin for degradation (Davis et al., 2003; Xiao et al., 2003). p120ctn contains multiple phosphorylation sites, the majority of which are in the N-terminal portion of the protein and expression of an N-terminal deleted mutant p120ctn blocks HGF-induced junctional breakdown in MDCK cells (Cozzolino et al., 2003). This suggests that regulation of phosphorylation of the N-terminal region might be involved in p120ctn-induced junctional breakdown, however it is not clear which phosphorylation sites could be involved or whether phosphorylation or dephosphorylation is important. The functional significance of individual p120ctn phosphorylation sites is currently the subject of intense study and the generation of phospho-specific p120 antibodies is currently underway (Vaughan et al., 2007). On first inspection, none of the serine or threonine phosphorylation sites in p120ctn appear to be potential PAK1 substrates comparing to the proposed PAK1 substrate consensus sequence (Rennefahrt et al., 2007). However, this does not rule out a role for PAK1 in direct or indirect control of p120ctn phosphorylation and junctional breakdown.

The role of PAK1 and PAK2 in cell:matrix adhesion signalling was investigated. FAK phosphorylation following HGF stimulation was not affected by PAK1 or PAK2 knockdown but confocal imaging revealed that PAK2 knockdown in DU145 cells led to an altered localisation of GIT1/2 and ERK1/2 in lamellipodia. ERK1/2 has been shown to localise to focal complexes following HGF stimulation in HeLa cells (Kermorgant et al., 2004) but this was not observed in DU145 cells where it localised to lamellipodia showing possible cell-specific differences. ERK1/2 is thought to play a role in the generation of new

focal adhesions by phosphorylating paxillin leading to the recruitment of FAK (Ishibe et al., 2003; Liu et al., 2002). This suggests that the large lamellipodia observed in PAK2-deficient cells could be caused by an increase in focal adhesion formation at the leading edge. It has recently been suggested that knockdown of PAK2 could lead to increased size of focal adhesions in heregulin-treated breast carcinoma cells (Coniglio et al., 2008), although only 20 cells were counted and only one oligomer of PAK2 siRNA was used. Future work should include measuring the size and number of focal adhesions in PAK2-deficient DU145 cells compared to control cells to investigate how ERK1/2 and GIT1/2 localisation affects focal adhesions in these conditions.

LIMK-mediated phosphorylation and inactivation of cofilin is thought to play a role in lamellipodia formation, but active, dephosphorylated cofilin is also believed to function at the leading edge, probably in the generation of new barbed ends by actin filament severing (Delorme et al., 2007). The phosphorylation of cofilin following HGF stimulation in PC3 cells has recently been described and it was suggested that this could occur through phosphorylation of LIMK1 by PAK4 (Ahmed et al., 2008) PAK1 and PAK2 have also both been described to phosphorylate LIMK1 and LIMK2 (Bokoch, 2003; Edwards et al., 1999; Misra et al., 2005). A phospho-LIMK antibody was tested but results were not conclusive because of the presence of many non-specific bands (data not shown). A phospho-cofilin antibody was also tested but did not detect a change in phosphorylation downstream of HGF in a preliminary experiment (data not shown). This could be because HGF stimulation was carried out in growth medium. If the cells were starved to decrease the background level of growth factor signalling, a change in cofilin phosphorylation might be observed and this could be tested in future experiments.

It is thought that PAK can also regulate actin polymerisation and membrane ruffle formation through filaminA; an actin-binding protein that induces cross-linking of F-actin into bundles (Grazi et al., 1990). FilaminA binds to PAK1 via a region overlapping the CRIB domain and is thought to induce PAK activity in the same way as Rac1/Cdc42 binding (Vadlamudi 2002). PAK can also phosphorylate filamin A at Ser2152, the relevance of which is unclear but is thought to be important for cell migration (Vadlamudi et al., 2002; Woo et al., 2004). In the present study the role of filamin A was not investigated in DU145 or PC3 cell migration but this could be included in future work.

Inhibition of HGF-induced cell rounding in PAK1-deficient DU145 cells (Chapter 4) could be due to an inhibition of cell contractility. PAK1 or PAK2 knockdown had no effect on the level of MLC phosphorylated at T18/S19 and there was also no change upon HGF stimulation for up to 1 hour in DU145 or PC3 cells. R-MLC is diphosphorylated at T18 and S19 by MLCK (Itoh et al., 1992). PAK1 and PAK2 have both been shown to phosphorylate MLCK *in vitro* (Goeckeler et al., 2000; Sanders et al., 1999) and constitutively active PAK1 induces MLCK phosphorylation when overexpressed (Sanders et al., 1999). Phosphorylation inactivates MLCK and would be expected to lead to a decrease in diphosphorylated R-MLC. The absence of a change in diphosphorylated R-MLC after HGF stimulation or in PAK1 or PAK2-deficient cells suggests that PAK1 and PAK2 do not phosphorylate MLCK following activation by HGF in DU145 and PC3 cells. However, a role in cell contractility in this context cannot be ruled out as monophosphorylation of MLC could also be important. It has previously been shown that PAK1 and PAK2 phosphorylate R-MLC at serine 19 *in vitro* (Chew et al., 1998; Ramos et al., 1997) and an increase in monophosphorylated R-MLC at S19 was also observed with overexpression of PAK2 or constitutively active PAK1 (Sells et al., 1999; Zeng et al., 2000). Future experiments should investigate whether R-MLC monophosphorylation at S19 is affected by HGF stimulation or by PAK1 or PAK2 knockdown. It would also be interesting to investigate the effect of PAK knockdown on phosphorylation of MLCK.

The role of PAKs in affecting microtubule regulation was investigated. Op18 became phosphorylated at Serine 16 following HGF stimulation in DU145 and PC3 cells. Phosphorylation of Op18 at this site inhibits its microtubule destabilisation ability and PAK has previously been shown to phosphorylate serine 16 *in vitro* (Wittmann et al., 2004). Knockdown of PAK1 in either cell line had no effect on Op18 phosphorylation but knockdown of PAK2 impaired Op18 phosphorylation in PC3 cells suggesting that PAK2 can phosphorylate Op18 at Ser 16, which has not been previously described. It is possible that impaired Op18 phosphorylation plays a role in the slower migration speed of PAK2-depleted PC3 cells. Interestingly, PAK2 knockdown had no effect on Op18 phosphorylation in DU145 cells. The difference could be due to a higher level of expression of PAK1 in DU145 cells compared to PC3 cells (Chapter 3, Figure 3.2). Therefore if both PAK1 and PAK2 can phosphorylate Op18, PAK1 could compensate for loss of PAK2 in DU145 cells but not in PC3 cells. If this was the case, it would be expected

that knocking down PAK1 and PAK2 simultaneously would lead to an inhibition of Op18 phosphorylation following HGF stimulation in both PC3 and DU145 cells. The observation that either Rac1 or Cdc42 knockdown affect Op18 phosphorylation agrees with this as they affect both PAK1 and PAK2 activation (Chapter 3.8). However, PAK2 knockdown appeared to affect microtubule organisation in DU145 cells despite the lack of an effect on phosphorylation Op18. This could be due to localised differences in Op18 phosphorylation or to another signalling pathway leading from PAK2 to microtubules. In future work it would be interesting to investigate this in more detail by investigating whether PAK knockdown leads to localised differences in the level of acetylated (more stable) or tyrosinated (less stable) α -tubulin (Witte et al., 2008)

Akt was rapidly phosphorylated at S473 in DU145 and PC3 cells following HGF stimulation and was unaffected by knockdown of PAK1 or PAK2. This suggests that the phosphorylation of Akt by PAK is not important in stimulation of cell scattering and migration by HGF. Instead, other kinases such as protein kinase C (PKC) or mammalian target of rapamycin complex 2 (mTORC2) are likely to be involved in phosphorylation of Akt at S473 (Guertin et al., 2006; Kawakami et al., 2004). PAK1 could be activated by Akt downstream of HGF. It has previously been shown that PAK1 is activated by expression of active Akt but not inactive Akt in COS7 cells (Menard and Mattingly, 2004) and Akt is thought to activate PAK1 in a Rho GTPase-independent manner (Tang et al., 2000). Akt activation involves targeting to the membrane via its PH-domain which binds PIP3 generated by PI3K. Subsequent phosphorylation of Akt at S473 and T308 leads to full activation (Reviewed in (Manning and Cantley, 2007)). This provides a possible mechanism by which Akt could lead to localised PAK activation. In the future work it would be interesting to study this in more detail by investigating PAK1 or PAK2 phosphorylation in Akt-deficient cells.

Investigation of MAPK signalling following HGF stimulation showed that the Raf/MEK/ERK pathway and p38 MAPK phosphorylation were not affected by knockdown of PAK1 or PAK2 in DU145 or PC3 cells. This is probably due to the many diverse signalling pathways that can feed into MAPK signalling. For example Ras-GTP binding to Raf regulates Raf activation (Avruch et al., 2001; Chong et al., 2003) and p38 MAPK can be activated by a diverse range of upstream MAP kinases (reviewed in Zarubin and Han,

2005). The observation that MEK phosphorylation did not change following HGF stimulation but both Raf1 and ERK1/2 became phosphorylated further highlights the diversity of MAPK signalling. Another way of investigating MAPK phosphorylation following HGF stimulation specifically would be to serum-starve the cells prior to HGF addition. The absence of other growth factors might attenuate other signalling pathways which could otherwise mask the effect of PAK knockdown.

In summary, phosphorylation of most of the known PAK1 and PAK2 substrates tested was not affected by PAK1 or PAK2 knockdown, probably due to the presence of other signalling pathways or PAK isoforms. Op18/stathmin phosphorylation was impaired by PAK2 knockdown in PC3 cells but not in DU145 cells which could be due to functional redundancy between PAK1 and PAK2, but PAK2 did seem to affect microtubule organisation in unstimulated cells revealing an interesting subject for future investigation. The effect of PAK2 on Op18/stathmin could explain the slower migration speed of PC3 cells but the same was not observed with PAK1 knockdown showing that there is probably a difference in signalling or localisation that was not tested in this study.

Chapter 6: Concluding remarks

In this study it was shown that PAK1 and PAK2 became phosphorylated following HGF stimulation in DU145, PC3, MCF7 and HT29 cancer cell lines. Phosphorylation in DU145 and HT29 cells was weak. This phosphorylation was partially abrogated by Rac1 or Cdc42 knockdown in DU145 and PC3 prostate cancer cells. PAK1 or PAK2 knockdown were shown to give distinct phenotypes in DU145 and PC3 cells but phosphorylation of many of the previously identified PAK targets was not affected. Possible negative regulation of PAK1 by PAK2 was observed as knockdown of PAK2 in both PC3 and DU145 cells led to an up regulation of phosphorylation of PAK1 at Ser199/204 by an unknown mechanism. Op18 was identified as a likely substrate of PAK2 which has not been previously described.

It has formerly been shown that overexpression of dominant negative or constitutively active PAK1 or Rho GTPases often has a strong effect on cell motility. For example dominant negative PAK1 or Cdc42 inhibited CXCL1-induced chemotaxis (Dharmawardhane et al., 1999); either dominant negative or constitutively active PAK1 inhibited endothelial cell migration (Kiosses et al., 1999); and dominant negative Rac1 blocked macrophage migration towards CSF-1 (Allen et al., 1998). The results from the present study agree with previous results to some extent but suggest that in some cases the phenotype associated with knockdown of a protein is not as strong as that associated with overexpression of a dominant negative. For example, preliminary movies of Rac1-depleted DU145 or PC3 cells showed that cells could still migrate and in the future these movies should be repeated and cell migration tracked to confirm this. One possible reason for the difference is that siRNA knockdown leaves a residual level of protein in the cell which could still function. The difference is also probably due to the binding and sequestering functions of dominant negative mutants and it is possible that in the future, siRNA might show that certain Rho GTPases or PAK isoforms do not have the same functional importance as previously described. An example of this can be seen in fibroblasts from Cdc42 knockout mice which can migrate and form filopodia and lamellipodia. Expression of dominant negative Cdc42 in these cells blocks migration showing that the mutant protein affects other signalling pathways (Czuchra et al., 2005). Furthermore, macrophages from Rac1 or Rac2 knockout mice have different morphology to those from control mice but, in contrast to previous studies with dominant negative Rac1, their migration speed is unaffected (Allen et al.,

1998; Wheeler et al., 2006). RNAi also makes it possible to distinguish between Rho GTPases where it was not previously possible. For example RhoA, RhoB and RhoC have been studied together by inhibition with C3 transferase but siRNA allows distinction between the three. It would be interesting to use combinations of siRNA to investigate signalling networks and compensation or feedback pathways between proteins.

Knockdown of PAK1 or PAK2 gave distinct phenotypes for which there are several possible explanations: PAK1 and PAK2 could have one or more unshared substrates leading to the activation of different downstream pathways; their cellular localisation could be differentially regulated or they could have one or more unshared upstream activators meaning their activity could be locally regulated in different parts of the cell. It has previously been suggested that the group I PAKs have almost identical substrate specificity *in vitro* although it is not known whether this is the case *in vivo* (Rennefahrt et al., 2007). It is possible that specific residues around the substrate phosphorylation site could confer selectivity between PAK1 and PAK2. For example in the Rennefahrt study, methionine at position -3 to the phosphorylatable serine in the substrate peptide seemed to completely block phosphorylation by PAK2 but not PAK1. Therefore the possibility that different group I PAKs have some unshared substrates cannot be ruled out. Results from the present study support the idea that many PAK1/2 substrates are shared but some are unique to one PAK isoform. PAK2 knockdown impaired Op18 phosphorylation in PC3 cells, but not in DU145 cells where more PAK1 is expressed. This is interesting as PAK1 has been shown to phosphorylate Op18 *in vitro* but the same result was not observed in cells (Wittmann et al., 2004). Knockdown of either Rac1 or Cdc42 which both activate PAK1 and PAK2, impaired Op18 phosphorylation in DU145 cells. Taken together these results suggest that both PAK1 and PAK2 can phosphorylate Op18 in cells but it would be interesting to confirm this using cells depleted in both PAK1 and PAK2. Phosphorylation of many of the known PAK substrates was unaffected by knockdown of PAK1 or PAK2. This could be due to functional redundancy between PAKs but it is also likely to be due to compensation through other pathways. For example ROCK can also lead to cofilin phosphorylation through LIMK (Sumi et al., 2001).

Some evidence that PAK2 could have a unique substrate comes from the observation that total endogenous Op18 phosphorylation was unaffected in PAK1 or PAK2-depleted DU145

cells, but PAK2 knockdown did appear to affect microtubule organisation whereas PAK1 knockdown did not. This effect could either be due to localised, isoform-specific PAK activity or a PAK2-specific substrate or signalling pathway affecting microtubule stability or dynamics. Little is known about which other microtubule-stabilising or destabilising proteins could be downstream of PAKs. PAK1 phosphorylates tubulin cofactor B (TCoB) and this is thought to be important for TCoB function in microtubule polymerisation (Vadlamudi et al., 2005) but so far no link has previously been published between PAK2 and microtubules. Future work could investigate whether PAK2 knockdown exerts its effect through localized alterations in stathmin activity or through a PAK2-specific substrate.

Perhaps the most striking evidence that PAK1 and PAK2 could have some unshared substrates is the observation that PAK2 knockdown led to an increase in PAK1 phosphorylation at S199/204 whereas PAK1 knockdown had no effect on PAK2 phosphorylation. S199/204 has been shown to be an autophosphorylation site (Gatti et al., 1999; Manser et al., 1997) so if PAK1 and PAK2 had identical substrate specificity they would be expected to *trans*-phosphorylate. The hypothesis would then be that PAK1 or PAK2 knockdown would lead to a decrease in phosphorylation of both isoforms but this was not the case. In addition, if PAK1 and PAK2 could heterodimerise it might be expected that knockdown of either one isoform would lead to an increase in phosphorylation of the other isoform. This was not observed, suggesting that PAK1 and PAK2 do not heterodimerise. Alignment of the PAK1 and PAK2 amino acid sequences surrounding the S199/204 phosphorylation sites shows that they are highly homologous but there are differences that could confer a difference in binding affinity to PAK1 or PAK2, although the non-homologous residues share similar characteristics (Figure 6.1). As yet no direct comparison has been made between the activity of PAK1 or PAK2 towards known substrates. It would be interesting to conduct an *in vitro* phosphorylation assay to compare PAK1 and PAK2-mediated phosphorylation of peptides surrounding the PAK1/2 autophosphorylation sites. It is likely that the phosphorylation of T423 in the kinase domain of PAK1 and PAK2 can be *trans*-phosphorylated between different isoforms. The region surrounding T423 is 100% identical between PAK1, PAK2 and PAK3 (Chapter 3, Figure 3.19) and it has been suggested that the crystal structure of active PAK1 reveals possible residues involved in dimerisation of the kinase domain (Pirrucello et al., 2006). These

residues are conserved between the group I PAKs but their relevance to phosphorylation at S199/204 is unclear as they are outside the kinase domain.

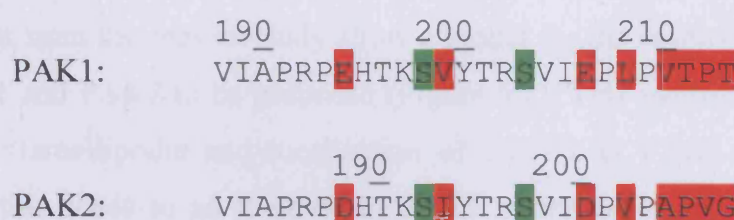


Figure 6.1: sequence alignment of PAK1 and PAK2 surrounding the S199/204 autophosphorylation sites. Phosphorylation sites are highlighted green, non-homologous residues are highlighted red.

It would be interesting to investigate what the PAK2-specific substrates could be. They could include a kinase that directly phosphorylates PAK1 at S199/204 or a phosphatase that dephosphorylates those sites. Very little is known about phosphatases which could regulate PAK dephosphorylation. So far only partner of PIX (POPX) 1 and 2 have been shown to deactivate PAK1 by dephosphorylation of T423 (Koh et al., 2002). It is not known whether this also corresponds to dephosphorylation of S199/204 but it is possible as they are both autophosphorylation sites (Manser et al., 1997). It would be interesting to find out whether POPX can be activated by PAK2.

Preliminary investigations into whether up-regulated PAK1 phosphorylation was responsible for the larger lamellipodia seen in PAK2-deficient DU145 cells were carried out by simultaneous knockdown of PAK1 and PAK2. Results were unclear due to variation between the phenotypes but it appeared that PAK1 knockdown did not rescue the PAK2 phenotype. Further characterisation of PAK2-depleted DU145 cells showed that GIT1/2 was enriched at the leading edges of larger lamellipodia following HGF stimulation, although further work is required to quantify this. The same was not observed in the lamellipodia of control or PAK1-depleted cells. Interestingly, the GIT1/2 localisation in HGF-stimulated, PAK2-depleted DU145 cells was similar to that observed with expression of an S273D paxillin mutant in CHO-K1 cells (Nayal et al., 2006). In the same study it was shown that PAK1 can phosphorylate paxillin at Ser273 leading to an increase in GIT1 binding. It has also been suggested that GIT1 phosphorylation at Ser709 by PAK leads to increased binding to paxillin, although further evidence is required (Webb et al., 2006).

PAK1 and PAK2 localise with GIT through mutual binding of PIX. Phosphorylation of PAK1/2 at S199/204 decreases binding to PIX and this is believed to lead to increased Rac1 activation (see Chapter 1, Section 1.7.3.ii). These previous observations together with results from the present study allow a model for the control of lamellipodium formation by PAK1 and PAK2 to be proposed (Figure 6.2). This model could explain the generation of larger lamellipodia and localization of GIT1/2 in PAK2-depleted DU145 cells. PAK2-depletion leads to an increase in PAK1 phosphorylation at S199/204 meaning that less PAK1 would be bound to PIX. Knockdown of PAK2 should also lead to a drop in the amount of PIX bound to PAK2. These combined effects would mean that the level of PIX unbound from PAK was higher than in control cells. This could activate Rac1 to stimulate lamellipodium formation through WAVE as previously described (see Chapter 1 section 1.6.2.iii). PAK1 being unbound from PIX could also free PAK1 to signal directly to downstream effectors, some of which are thought to lead directly to the generation of lamellipodia. Phosphorylation of GIT1 or paxillin by PAK1, as previously described, could influence the localisation of GIT1/PIX to forming adhesions through increased binding to paxillin (see Chapter 1, section 1.7.3). Interestingly this model might also explain why PAK1 knockdown did not appear to rescue the PAK2 phenotype as the absence of both PAK1 and PAK2 could still lead to increased PIX-induced Rac1 activation and lamellipodium formation through an increase in PIX not bound to PAK.

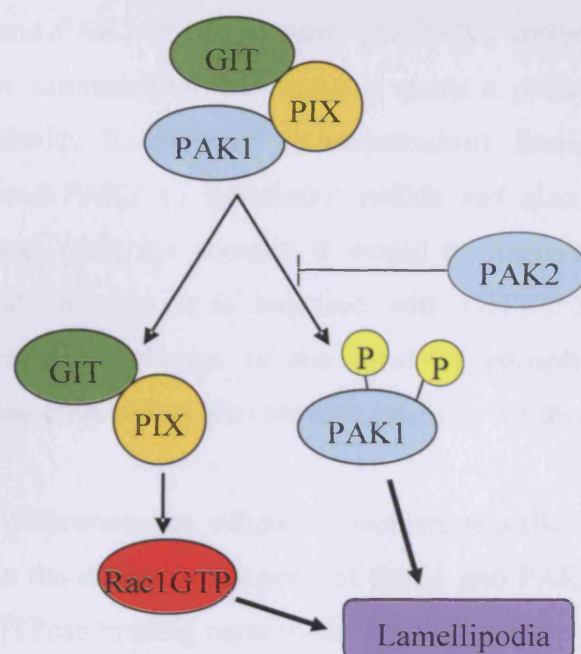


Figure 6.2: Hypothetical model for the control of lamellipodium formation by PAK1/2 in DU145 cells. Downstream of a stimulus, PAK1 would become phosphorylated and stimulate lamellipodium formation directly as previously described in the literature. PAK2 could inhibit this phosphorylation of PAK through an as yet unknown mechanism. PAK1 phosphorylation would free PIX to stimulate lamellipodium formation through Rac1.

Clearly further evidence is required to support this model. It would be important to determine the level and localisation of Rac1 activity in PAK1 or PAK2-depleted cells. The level of Rac1 activation could be easily determined in pull-down assays using the PAK PBD. Unfortunately a preliminary attempt to do this was inconclusive but in future the assay could be optimised to obtain a clear result. The localisation of Rac activity would be more challenging to observe; a fluorescent resonance energy transfer (FRET) probe to visualise Rac1 activity in cells has previously been developed (Itoh et al., 2002). The probe is composed of a YFP-tagged PAK1 CRIB domain linked to a CFP-tagged Rac1 molecule. Activation of the Rac1 in the probe leads to binding of the PAK1 CRIB which allows FRET to take place between the CFP donor and YFP acceptor. However, expression of the probe itself would probably affect cell morphology and motility. FRET could also be used to investigate interactions between PAK1/2, PIX, GIT1/2 and proteins that could localise these complexes to lamellipodia such as paxillin. However, this would also involve overexpression of tagged proteins which could affect cell morphology. If FRET was not successful, colocalisation of PIX with GIT1/2 and paxillin in PAK2-depleted cells could be investigated by immunofluorescence on fixed cells. It would also be helpful to know the

localisation of PAK1 and PAK2 in this system. The PAK1 antibody does not seem to be specific when used for immunofluorescence as it stains a protein in ruffles in PAK1^{-/-} mouse macrophages (Smith, S., personal communication). Preliminary results from the PAK2 antibody localised PAK2 to membrane ruffles and also gave punctate staining throughout the cytoplasm (data not shown). It would be interesting to test other PAK1 antibodies to investigate whether it is localised with GIT1/2 in PAK2-depleted cells following HGF treatment. A change in the level of phospho-paxillin or increased localisation to the leading edge would also provide evidence for this model.

As well as possible differences in substrate, isoform-specific localisation or activity probably plays a role in the different functions of PAK1 and PAK2. Most of the previous work to identify Rho GTPase binding partners for PAKs has focussed on PAK1 and as yet only Rac1 and Cdc42 have been shown to bind PAK2 but it is probable that others will be identified in future (See Table 1.1, Section 1.7.1.1). Comparison of the structural characteristics of PAK1 and PAK2 shows that PAK1 has three additional putative SH3-binding regions between the regulatory and kinase domains. Possible binding partners for these regions remain to be identified but their presence suggests that PAK1 might bind a different subset of adapter proteins than PAK2. It is also possible that PAK1/2 differ in their GTPase-independent activation; PAK2 is the only isoform that has been shown to be activated by cleavage (Rudel and Bokoch, 1997) but whether this plays any role other than in apoptosis is unknown. Also, so far only PAK1 has been shown to be activated by Filamin-A binding (Vadlamudi et al., 2002).

The role of PAK4 in the migration of DU145 and PC3 cells was not clear from this investigation. It has recently been shown that knockdown of PAK4 in PC3 cells leads to decreased migration of serum-starved cells in response to HGF (Ahmed et al., 2008), a finding not replicated by the present study using growing cells. This suggests that the role of PAK4 in PC3 cell migration may be specific to certain growth factor pathways and the loss of PAK4 could be compensated for in growing conditions. Results in DU145 cells suggested that PAK4 does play some role in cell migration or scattering but variation between oligomers meant that it was not possible to determine the role. In the future it would be interesting to test siRNA oligomers with a chemical modification to decrease off-target effects.

Results from PAK4 knockdown as well as PAK1 or PAK2 knockdown experiments highlight the problem of off-target effects associated with siRNA and the need for adequate controls. This is particularly evident in wound healing experiments in DU145 cells where oligomer PAK2b slowed wound healing significantly whereas all three other PAK2 oligomers had no effect (Figure 4.22). It also appears that oligomers that give a similar phenotype in one assay can give a significantly different phenotype in another. For example, oligomers PAK2b and PAK2c gave similar phenotypes in scattering assays but only oligomer PAK2c impaired migration through Matrigel. However, results from the observation of protein phosphorylation following HGF stimulation were consistent between the pairs of oligomers chosen suggesting that RNAi is a robust technique to study cell signalling. Therefore, the differences in morphological and functional phenotypes between siRNAs directed to a common target are likely to be due to the off-target knockdown of unrelated signalling pathways.

In this study, off-target effects were observed even though an siRNA concentration of 3.3 nM was used which is 15-30 fold lower than the concentrations used in many publications. This highlights the need to validate results by the use of multiple separate and different siRNA oligomers against a single target and argues against the use of pools of siRNA oligomers. Using a pool of oligomers is likely to multiply off-target effects and increase the risk of a false-positive phenotype unless each individual oligomer in the pool is titrated to such a low concentration that it would not decrease protein expression when used alone. This also has implications when knocking down two different proteins simultaneously i.e. the risk of an off-target phenotype is likely to increase with the number of different oligomers, used as observed in this study when PAK1 and PAK2 were knocked down together. Hopefully in the future, modification of siRNA to reduce off-target effects will make double-knockdown experiments more reliable.

As an alternative to using siRNA, the migration and morphology of cells isolated from PAK knockout mice could be investigated. This method also has a drawback in that compensation can occur where the gene expression profile might be different from control mice to knockout mice. This could be avoided by using the cyclisation recombination (Cre)/*LoxP* inducible knockout system where transgenic mice are generated with a Cre

gene under the control of an inducible promoter and *LoxP* recombination sites flanking the gene to be knocked out. Cre expression can then be induced in isolated cells *in vitro* leading to recombination of the *LoxP* sites and knockout of the gene of interest. However, using a knockout mouse does not allow the functions of a protein in human cancer cells to be investigated.

The potential roles for PAK3, PAK5 and PAK6 in cancer cell migration were not addressed in this study and will be an important subject for future work. PAK6 has been shown to be upregulated at the mRNA level in several cancer cell lines although this is not as frequent as PAK4 upregulation (Callow et al., 2002). PAK6 is believed to play a role in the inhibition of androgen receptor (AR) signalling (Lee et al., 2002; Schrantz et al., 2004). An early stage of prostate cancer is androgen-dependent growth, and inhibition of AR signalling is known to be an effective treatment at this early stage. This suggests that the upregulation of PAK6 in cancer is not relevant to progression of the disease. However, it is not known whether PAK6 plays another role. In the case of PAK5, it was suggested that it could be functionally redundant because the PAK5-null mouse had no detected abnormality, but emerging evidence implies that PAK5 activates Raf-1 and targets it to mitochondria (Li and Minden, 2003; Wu et al., 2008). PAK5 is also the only PAK that has been shown to bind RhoD and RhoH suggesting it is differentially regulated to the other PAKs (Wu and Frost, 2006). Whether PAK5 has a role in cancer is still unclear. PAK3 is highly homologous to the other group I PAKs and is thought to have similar substrate specificity implying that it could play a role in cancer cell migration if upregulated. It would be interesting to compare the expression of PAK3 in normal and cancerous tissue. If PAK3 overexpression occurs it would be a candidate for a role in cancer metastasis.

PAK isoforms have previously been shown to be upregulated in cancer (Callow et al., 2002) and the findings that the knockdown of PAK1 or PAK2 slows the migration speed of PC3 cells, and PAK1 depletion inhibits scattering of DU145 cells agree with the suggestion that they could be important in cancer metastasis. A possible direction for future work would be to investigate how depletion of PAK1 or PAK2 affect the migration of cells in three dimensions (3D) or in an *in vivo* model. The question of how two dimensional (2D) migration assays relate to cancer metastasis *in vivo* has been a subject of debate for years. It has been shown that the composition and organisation of cell:matrix adhesions differs

between 2D and *in vivo* and stress fibres have only been found in vascular endothelial cells *in vivo* (Cukierman et al., 2001; Wong et al., 1983). It has also been shown *in vivo* that cells can migrate and invade in a 3D environment in an amoeboid manner as well as a mesenchymal manner (reviewed in (Friedl and Wolf, 2003)). The need to study cell migration and invasion in a 3D setting has led to the development of *in vitro* assays in which cells are embedded or buried in a matrix often derived from the extracellular matrix of a mouse tumour (Lee et al., 2007). Furthermore, advances in multi-photon excitation fluorescence microscopy have made it possible to image tumour cells moving *in vivo* in mouse models (Wyckoff et al., 2006). Nevertheless, the study of 2D cell migration and cancer cell scattering provides a simple system to determine proteins of relevance to cell motility and to investigate cell signalling in detail.

In summary, in this project a novel regulatory pathway from PAK2 to PAK1 has been identified which clearly shows that PAK1 and PAK2 have independent functions. Differences between the effects of siRNA knockdown of PAK1 or PAK2 on cancer cell migration have been characterised which could be caused by a combination of isoform-specific protein localisation and substrate specificity.

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